

METHOD FOR DETECTION OF DRUG-SELECTED MUTATIONS  
IN THE HIV PROTEASE GENEd1  
5 1. FIELD OF THE INVENTION

The present invention relates to the field of HIV diagnosis. More particularly, the present invention relates to the field of diagnosing the susceptibility of an HIV sample to antiviral drugs used to treat HIV infection.

10 The present invention relates to a method for the rapid and reliable detection of drug-selected mutations in the HIV protease gene allowing the simultaneous characterization of a range of codons involved in drug resistance using specific sets of probes optimized to function together in a reverse-hybridization assay.

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2. BACKGROUND OF THE INVENTION

20 The human immunodeficiency virus (HIV) is the ethiological agent for the acquired immunodeficiency syndrome (AIDS). HIV, like other retroviruses, encodes an aspartic protease that mediates the maturation of the newly produced viral particle by cleaving viral polypeptides into their functional forms (Hunter *et al.*). The HIV protease is a dimeric molecule consisting of two identical subunits each contributing a catalytic aspartic residue (Navia *et al.*, Whodawer *et al.*, Meek *et al.*). Inhibition of this enzyme gives rise to noninfectious viral particles that cannot establish new cycles of viral replication (Kohl *et al.*, Peng *et al.*).

25 Attempts to develop inhibitors of HIV-1 protease were initially based on designing peptide compounds that mimicked the natural substrate. The availability of the 3-dimensional structure of the enzyme have more recently allowed the rational design of protease inhibitors (PI) using computer modeling (Huff *et al.*, Whodawer *et al.*). A number of second generation PI that are partially peptidic or entirely nonpeptidic have proven to exhibit particularly potent antiviral effects in cell culture.

30 Combinations of various protease inhibitors with nucleoside and non-nucleoside RT inhibitors have also been studied extensively *in vitro*. In every instance, the combinations have been at least additive and usually synergistic.

35 In spite of the antiviral potency of many recently developed HIV-1 PI, the emergence of virus variants with decreased sensitivity to these compounds has been described both in cell culture and in treated patients thereby escaping the inhibitory effect of the antiviral (Condra *et al.*). Emergence of

resistant variants depends on the selective pressure applied to the viral population. In the case of a relatively ineffective drug, selective pressure is low because replication of both wild-type virus and any variants can continue. If a more effective drug suppresses replication of virus except for a resistant variant, then that variant will be selected. Virus variants that arise from selection by PI carry several distinct mutations in the protease coding sequence that appear to emerge sequentially. A number of these cluster near the active site of the enzyme while others are found at distant sites. This suggests conformational adaptation to primary changes in the active site and in this respect certain mutations that increase resistance to PI also decrease protease activity and virus replication.

Amongst the PI, the antiviral activity of the PI ritonavir (A-75925; ABT-538), nelfinavir (AG-1343), indinavir (MK-639; L735; L524) and saquinavir (Ro 31-8959) have been approved by the Food and Drug Administration and are currently under evaluation in clinical trials involving HIV-infected patients. The VX-487 (141W94) antiviral compound is not yet approved. The most important mutations selected for the above compounds and leading to gradually increasing resistance are found at amino acid (aa) positions 30 (D to N), 46 (M to I), 48 (G to V), 50 (I to V), 54 (I to A, I to V), 82 (V to A, or F, or I, or T), 84 (I to V) and 90 (L to M). Other mutations associated with drug resistance to the mentioned compounds have been described (Schinazi *et al*). Saquinavir-resistant variants, which usually carry mutations at amino acid positions 90 and/or 48, emerge in approximately 45% of patients after 1 year of monotherapy. Resistance appears to develop less frequently with higher doses of saquinavir. Resistance to indinavir and ritonavir requires multiple mutations; usually at greater than 3 and up to 11 sites, with more amino acid substitutions conferring higher levels of resistance. Resistant isolates usually carry mutations at codons 82, 84, or 90. In the case of ritonavir, the mutation at codon 82 appears first in most patients. Although mutant virions resistant to saquinavir are not cross-resistant to indinavir or ritonavir, isolates resistant to indinavir are generally ritonavir resistant and visa versa. Resistance to either indinavir or ritonavir usually results in cross-resistance to saquinavir. Approximately one third of indinavir resistant isolates are cross-resistant to nelfinavir as well.

The regime for an efficient antiviral treatment is currently not clear at all. Patterns of reduced susceptibility to HIV protease inhibitors have been investigated *in vitro* by cultivating virus in the presence of PI. These data, however, do not completely predict the pattern of amino-acid changes actually seen in patients receiving PI. Knowledge of the resistance and cross-resistance patterns should facilitate selection of optimal drug combinations and selection of sequences with non-overlapping resistance patterns. This would delay the emergence of cross-resistant viral strains and prolong the duration of effective antiretroviral activity in patients. Therefore, there is need for methods and systems that detect these mutational events in order to give a better insight into the mechanisms of HIV resistance. Further, there is need for methods and systems which can provide data important for the antiviral therapy to follow in a more time-efficient and economical manner compared to the conventional cell-culture selection techniques.

### 3. AIMS OF THE INVENTION

It is an aim of the present invention to develop a rapid and reliable detection method for  
5 determination of the antiviral drug resistance of viruses, which contain protease genes such as HIV retroviruses present in a biological sample.

More particularly it is an aim of the present invention to provide a genotyping assay allowing the detection of the different HIV protease gene wild type and mutation codons involved in the antiviral resistance in one single experiment.

10 It is also an aim of the present invention to provide an HIV protease genotyping assay or method which allows to infer the nucleotide sequence at codons of interest and/or the amino acids at the codons of interest and/or the antiviral drug selected spectrum, and possibly also infer the HIV type or subtype isolate involved.

15 Even more particularly it is an aim of the present invention to provide a genotyping assay allowing the detection of the different HIV protease gene polymorphisms representing wild-type and mutation codons in one single experimental setup.

It is another aim of the present invention to select particular probes able to discriminate wild-type HIV protease sequences from mutated or polymorphic HIV protease sequences conferring resistance to one or more antiviral drugs, such as ritonavir (A-75925; ABT-538), nelfinavir (AG-1343), indinavir  
20 (MK-639; L735; L524), saquinavir (Ro 31-8959) and VX-478 (141W94) or others (Shinazi *et al*).

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35 resistance to VX-478 (141W94).

It is also an aim of the present invention to select particular probes able to determine and/or infer

cross-resistance to HIV protease inhibitors.

It is more particularly an aim of the present invention to select particular probes able to discriminate wild-type HIV protease from mutated HIV protease sequences involving at least one of amino acid positions 30 (D to N), 46 (M to I), 48 (G to V), 50 (I to V), 54 (I to A or V), 82 (V to A or 5 F or I or T), 84(I to V) and 90 (L to M) of the viral protease gene.

It is particularly an aim of the present invention to select a particular set of probes, able to discriminate wild-type HIV protease sequences from mutated HIV protease sequences conferring resistance to any of the antiviral drugs defined above with this particular set of probes being used in a reverse hybridization assay.

10 It is moreover an aim of the present invention to combine a set of selected probes able to discriminate wild-type HIV protease sequences from mutated HIV protease sequences conferring resistance to antiviral drugs with another set of selected probes able to identify the HIV isolate, type or subtype present in the biological sample, whereby all probes can be used under the same hybridization and wash-conditions.

15 It is also an aim of the present invention to select primers enabling the amplification of the gene fragment(s) determining the antiviral drug resistance trait of interest.

It is also an aim of the present invention to select particular probes able to identify mutated HIV protease sequences resulting in cross-resistance to antiviral drugs.

20 The present invention also aims at diagnostic kits comprising said probes useful for developing such a genotyping assay.

The present invention also aims at diagnostic kits comprising said primers useful for developing such a genotyping assay.

25 **4. DETAILED DESCRIPTION OF THE INVENTION.**

All the aims of the present invention have been met by the following specific embodiments.

According to one embodiment, the present invention relates to a method for determining the susceptibility to antiviral drugs of HIV viruses in a biological sample, with said method comprising:

30 a) if need be, releasing, isolating or concentrating the polynucleic acids present in the sample;  
b) if need be amplifying the relevant part of the protease gene of HIV with at least one suitable primer pair;  
c) hybridizing the polynucleic acids of step a) or b) with at least one of the following probes:

probes specifically hybridizing to a target sequence comprising codon 30;

35 probes specifically hybridizing to a target sequence comprising codon 46 and/or 48;

probes specifically hybridizing to a target sequence comprising codon 50;

probes specifically hybridizing to a target sequence comprising codon 54;  
probes specifically hybridizing to a target sequence comprising codon 82 and/or 84;  
probes specifically hybridizing to a target sequence comprising codon 90;  
or the complement of said probes,

- 5 further characterized in that said probes specifically hybridize to any of the target sequences presented in figure 1, or to the complement of said target sequences;  
d) inferring from the result of step c) whether or not a mutation giving rise to drug resistance is present in any of said target sequences.

The numbering of HIV-1 protease gene encoded amino acids is as generally accepted in literature.

- 10 Mutations that give rise to an amino acid change at position 48 or 90 are known to confer resistance to saquinavir (Erlebe *et al*; Tisdale *et al*). An amino acid change at codon 46 or 54 or 82 or 84 results in ritonavir and indinavir resistance (Kempf *et al*; Emini *et al*; Condra *et al*). Amino acid changes at positions 30 and 46 confer resistance to nelfinavir (Patock *et al*) and amino acid changes at position 50 confers resistance to VX-487 (Rao *et al*). Therefore, the method described above allows to determine  
15 whether a HIV strain is susceptible or resistant to any of the drugs mentioned above. This method can be used, for instance, to screen for mutations conferring resistance to any of the mentioned drugs before initiating therapy. This method may also be used to screen for mutations that may arise during the course of therapy (i.e. monitoring of drug therapy). It is obvious that this method may also be used to determine resistance to drugs other than the above-mentioned drugs, provided that resistance to these other drugs  
20 is linked to mutations that can be detected by use of this method. This method may also be used for the specific detection of polymorphic nucleotides. It is to be understood that the said probes may only partly overlap with the targets sequences of figure 1, table 2 and table 3, as long as they allow for specific detection of the relevant polymorphic nucleotides as indicated above. The sequences of figure 1, table 2 and table 3 were derived from polynucleic acid fragments comprising the protease gene. These  
25 fragments were obtained by PCR amplification and were inserted into a cloning vector and sequence analyzed as described in example 1. It is to be noted that some polynucleic acid fragments comprised polymorphic nucleotides in their sequences, which have not been previously disclosed. These novel polymorphic nucleotide sequences are represented in table 4 below.

30 TABLE 4: Polymorphic nucleotide sequences.

51	52	53	54	55	56	57	58	codon position	
gga	ggt	ttt	atc	aaa	gtt	aga	cag	consensus sequence	
GGA	GGT	TTT	ATC	AAA	GTC	AGA	CAA	SEQ ID NO 478	
35	GGA	GGT	TTC	ATT	AAG	GTA	AAA	CAG	SEQ ID NO 479
	GGA	GGT	TTT	ATT	AAG	GTA	AGA	CAG	SEQ ID NO 480

GGA GGT TTT ATT AAA GTA AGA CAA	SEQ ID NO 481
GGA GGC TTT ATC AAA GTA AGA CAA	SEQ ID NO 482
GGA GGT TTT ATC AAA GTC AGA CAA	SEQ ID NO 483

5      78    79    80    81    82    83    84    85	codon position
gga cct aca cct gtc aac ata att gg	consensus sequence
GGA CCT ACA CCA GTC AAC ATA ATT GG	SEQ ID NO 484
GGA CCT ACA CCT GCC AAT ATA ATT GG	SEQ ID NO 485
GGA CCT ACG CCC TTC AAC ATA ATT GG	SEQ ID NO 486
10     GGA CCG ACA CCT GTC ACC ATA ATT GG	SEQ ID NO 487
GGA CCT ATA CCT GTC AAC ATA ATT GG	SEQ ID NO 488

87    88    89    90    91    92    93    94	codon position
a aga aat ctg ttg act cag att ggc	consensus sequence
A AAA AAT CTG ATG ACT CAG ATT GGC	SEQ ID NO 489
A AGA ACT CTG TTG ACT CAG CTT GGA	SEQ ID NO 490
A AGA AAT ATG ATG ACC CAG CTT GGC	SEQ ID NO 491
A AGA AAT ATA ATG ACT CAG CTT GGA	SEQ ID NO 492
A AGA AAT CTG CTG ACT CAG ATT GGG	SEQ ID NO 493
A AGA AAT CTG TTG ACA CAG CTT GGC	SEQ ID NO 494
A AGA AAT ATG TTG ACT CAG CTT GGT	SEQ ID NO 495
A AGA AAT TTG TTG ACT CAG ATT GGG	SEQ ID NO 496
A AGA AAT ATG TTG ACT CAG CTT GGT	SEQ ID NO 497
A AGA AAT ATG TTG ACT CAG CTT GGA	SEQ ID NO 498
A AGA AAT CTG TTG ACT CAG CTT GGA	SEQ ID NO 499
A AGA AAC CTG TTG ACT CAA CTT GGT	SEQ ID NO 500

The present invention thus also relates to these novel sequences, or a fragment thereof, wherein said  
15 fragment consists of at least 10, preferably 15, even more preferably 20 contiguous nucleotides and  
contains at least one polymorphic nucleotide. It is furthermore to be understood that these new

polymorphic nucleotides may also be expected to arise in another sequence context than in the mentioned sequences. For instance a G at the third position of codon 55 is shown in SEQ ID N° 478 in combination with a T at the third position of codon 54, but a G at the third position of codon 55 may also be expected to occur in the context of a wild type sequence. It is also to be understood that the above 5 mentioned specifications apply to the complement of the said target sequences as well. This applies also to Figure 1.

According to a preferred embodiment the present invention relates to a method as indicated above, further characterized in that said probes are capable of simultaneously hybridizing to their respective target regions under appropriate hybridization and wash conditions allowing the detection of 10 the hybrids formed.

According to a preferred embodiment, step c is performed using a set of at least 2, preferably at least 3, more preferably at least 4 and most preferably at least 5 probes meticulously designed as such that they show the desired hybridization results. In general this method may be used for any purpose that relies on the presence or absence of mutations that can be detected by this method, e.g. for genotyping. 15 The probes of table 1 have been optimized to give specific hybridization results when used in a LiPA assay (see below), as described in examples 2 and 3. These probes have thus also been optimized to simultaneously hybridize to their respective target regions under the same hybridization and wash conditions allowing the detection of hybrids. The sets of probes for each of the codons 30, 46/48, 50, 54 and 82/84 have been tested experimentally as described in examples 2 and 3. The reactivity of the 20 sets shown in table 1 with 856 serum samples from various geographic origins was evaluated. It was found that the sets of probes for codons 30, 46/48, 50, 54 and 82/84 reacted with 98.9%, 99.6%, 98.5%, 99.2%, 95.4% and 97.2% of the test samples, respectively. The present invention thus also relates to the sets of probes for codons 30, 46/48, 50, 54, 82/84 and 90, shown in table 1 and table 7.

According to another even more preferred embodiment, the present invention relates to a method 25 as defined above, further characterized in that:

step b) comprises amplifying a fragment of the protease gene with at least one 5'-primer specifically hybridizing to a target sequence located between nucleotide position 210 and nucleotide position 260 (codon 87), more preferably between nucleotide position 220 and nucleotide position 260 (codon 87), more preferably between nucleotide position 230 and 30 nucleotide position 260 (codon 87), even more preferably at nucleotide position 241 to nucleotide position 260 (codon 87) in combination with at least one suitable 3'- primer, and

step c) comprises hybridizing the polynucleic acids of step a) or b) with at least one of the probes specifically hybridizing to a target sequence comprising codon 90.

35 According to another even more preferred embodiment, the present invention relates to a method as defined above, further characterized in that:

- step b) comprises amplifying a fragment of the protease gene with at least one 3'-primer specifically hybridizing to a target sequence located between nucleotide position 253 (codon 85) and nucleotide positions 300, more preferably between nucleotide position 253 (codon 85) and nucleotide positions 290, more preferably between nucleotide position 253 (codon 85)
- 5 and nucleotide positions 280, even more preferably at nucleotide position 253 (codon 85) to nucleotide position 273 (codon 91), in combination with at least one suitable 5'-primer, and
- step c) comprises hybridizing the polynucleic acids of step a) or b) with at least one of the probes specifically hybridizing to a target sequence comprising any of codons 30, 46, 48, 50, 52, 54, 82 and 84.
- 10 It has been found, unexpectedly, that an amplified nucleic acid fragment comprising all of the above-mentioned codons, does not hybridize optimally to probes comprising codon 82, 84 or 90. On the other hand, a shorter fragment, for instance the fragment which is amplified by use of the primers Prot41bio and Prot6bio with respectively seq id no 5 and seq id no 4, hybridizes better to probes comprising codon 90. Better hybridization is also obtained when the fragment is amplified with primer Prot41bio in
- 15 combination with primers Prot6abio, Prot6bbio, Prot6cbio and Prot6dbio. The present invention thus also relates to a method as defined above, further characterized in that the 5'-primer is seq id no 5 and at least one 3' primer is chosen from seq id no 4, seq id no 506, seq id no 507, seq id no 508, and seq id no 509. Likewise, another shorter fragment, for instance the fragment which is amplified by use of the primers Prot2bio and Prot31bio with respectively seq id no 3 and seq id no 6, was found to hybridize better to
- 20 probes comprising codon 82 and/or 84. Hence the present invention also relates to a method as defined above, further characterized in that the 5'-primer is seq id no 5 and at least one 3'-primer is chosen from seq id no 4, seq id no 506, seq id no 507, seq id no 508, and seq id no 509..
- New sets of amplification primers as mentioned in example 1 were selected. The present invention thus also relates to primers: prot 16 (SEQ ID NO 501), prot 5 (SEQ ID NO 5), prot2a bio (SEQ ID NO 503),
- 25 prot2b bio (SEQ ID NO 504), prot31 bio (SEQ ID NO 6), prot41-bio (SEQ ID NO 505), prot6a (SEQ ID NO 506), prot6b (SEQ ID NO 507), prot6c (SEQ ID NO 508) and prot6d (SEQ ID NO 509). A number of these primers are chemically modified (biotinylated), others are not. The present invention relates to any of the primers mentioned, primers containing unmodified nucleotides, or primers containing modified nucleotides.
- 30 Different techniques can be applied to perform the sequence-specific hybridization methods of the present invention. These techniques may comprise immobilizing the amplified HIV polynucleic acids on a solid support and performing hybridization with labeled oligonucleotide probes. HIV polynucleic acids may also be immobilized on a solid support without prior amplification and subjected to hybridization. Alternatively, the probes may be immobilized on a solid support and hybridization may
- 35 be performed with labeled HIV polynucleic acids, preferably after amplification. This technique is called reverse hybridization. A convenient reverse hybridization technique is the line probe assay (LiPA). This

assay uses oligonucleotide probes immobilized as parallel lines on a solid support strip (Stuyver et al., 1993). It is to be understood that any other technique based on the above-mentioned methods is also covered by the present invention.

According to another preferred embodiment, the present invention relates to any of the probes mentioned above and/or to any of the primers mentioned above, with said primers and probes being designed for use in a method for determining the susceptibility to antiviral drugs of HIV viruses in a sample. According to an even more preferred embodiment, the present invention relates to the probes with seq id no 7 to seq id no 477 and seq id no 510 to seq id no 519, more preferably to the seq id no mentioned in Table 1 and Table 7, and to the primers with seq id no 3, 4, 5 and 6, 501, 502, 503, 504, 505, 506, 507, 508 and 509. The skilled man will recognize that addition or deletion of one or more nucleotides at their extremities may adapt the said probes and primers. Such adaptations may be required if the conditions of amplification or hybridization are changed, or if the amplified material is RNA instead of DNA, as is the case in the NASBA system.

According to another preferred embodiment, the present invention relates to a diagnostic kit enabling a method for determining the susceptibility to antiviral drugs of HIV viruses in a biological sample, with said kit comprising:

- a) when appropriate, a means for releasing, isolating or concentrating the polynucleic acids present in said sample;
- b) when appropriate, at least one of the primers of any of claims 4 to 6;
- c) at least one of the probes of any of claims 1 to 3, possibly fixed to a solid support;
- d) a hybridization buffer, or components necessary for producing said buffer;
- e) a wash solution, or components necessary for producing said solution;
- f) when appropriate, a means for detecting the hybrids resulting from the preceding hybridization;
- h) when appropriate, a means for attaching said probe to a solid support.

## DEFINITIONS

The following definitions serve to illustrate the terms and expressions used in the present invention.

The term "antiviral drugs" refers particularly to any antiviral protease inhibitor. Examples of such antiviral drugs and the mutation they may cause in the HIV protease gene are disclosed in Schinazi et al., 1997. The contents of the latter two documents particularly are to be considered as forming part of the present invention. The most important antiviral drugs focussed at in the present invention are disclosed in Tables 1 to 2.

The target material in the samples to be analyzed may either be DNA or RNA, e.g.: genomic DNA, messenger RNA, viral RNA or amplified versions thereof. These molecules are also termed polynucleic acids.

It is possible to use genomic DNA or RNA molecules from HIV samples in the methods according to the present invention.

Well-known extraction and purification procedures are available for the isolation of RNA or DNA from a sample (fi. in Maniatis et al., Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbour Laboratory Press (1989)).

The term "probe" refers to single stranded sequence-specific oligonucleotides, which have a sequence, which is complementary to the target sequence to be detected.

The term "target sequence" as referred to in the present invention describes the wild type nucleotide sequence, or the sequence comprising one or more polymorphic nucleotides of the protease gene to be specifically detected by a probe according to the present invention. This nucleotide sequence may encompass one or several nucleotide changes. Target sequences may refer to single nucleotide positions, codon positions, nucleotides encoding amino acids or to sequences spanning any of the foregoing nucleotide positions. In the present invention said target sequence often includes one or two variable nucleotide positions.

The term "polymorphic nucleotide" indicates a nucleotide in the protease gene of a particular HIV virus that is different from the nucleotide at the corresponding position in at least one other HIV virus. The polymorphic nucleotide may or may not give rise to resistance to an antiviral drug. It is to be understood that the complement of said target sequence is also a suitable target sequence in some cases. The target sequences as defined in the present invention provide sequences which should be complementary to the central part of the probe which is designed to hybridize specifically to said target region.

The term "complementary" as used herein means that the sequence of the single stranded probe is exactly the (inverse) complement of the sequence of the single-stranded target, with the target being defined as the sequence where the mutation to be detected is located.

"Specific hybridization" of a probe to a target sequence of the HIV polynucleic acids means that said probe forms a duplex with part of this region or with the entire region under the experimental conditions used, and that under those conditions said probe does not form a duplex with other regions of the polynucleic acids present in the sample to be analyzed.

Since the current application requires the detection of single basepair mismatches, very stringent conditions for hybridization are required, allowing in principle only hybridization of exactly complementary sequences. However, variations are possible in the length of the probes (see below), and it should be noted that, since the central part of the probe is essential for its hybridization characteristics, possible deviations of the probe sequence versus the target sequence may be allowable towards head and tail of the probe, when longer probe sequences are used. These variations, which may be conceived from the common knowledge in the art, should however always be evaluated experimentally, in order to check if they result in equivalent hybridization characteristics than the exactly complementary probes.

Preferably, the probes of the invention are about 5 to 50 nucleotides long, more preferably from about 10 to 25 nucleotides. Particularly preferred lengths of probes include 10, 11, 12, 13, 14, 15, 16, 17,

18, 19, 20, 21, 22, 23, 24 or 25 nucleotides. The nucleotides as used in the present invention may be ribonucleotides, deoxyribonucleotides and modified nucleotides such as inosine or nucleotides containing modified groups, which do not essentially alter their hybridization characteristics.

5 Probe sequences are represented throughout the specification as single stranded DNA oligonucleotides from the 5' to the 3' end. It is obvious to the man skilled in the art that any of the below-specified probes can be used as such, or in their complementary form, or in their RNA form (wherein U replaces T).

10 The probes according to the invention can be prepared by cloning of recombinant plasmids containing inserts including the corresponding nucleotide sequences, if need be by cleaving the latter out from the cloned plasmids upon using the adequate nucleases and recovering them, e.g. by fractionation according to molecular weight. The probes according to the present invention can also be synthesized chemically, for instance by the conventional phospho-triester method.

15 The term "solid support" can refer to any substrate to which an oligonucleotide probe can be coupled, provided that it retains its hybridization characteristics and provided that the background level of hybridization remains low. Usually the solid substrate will be a microtiter plate, a membrane (e.g. nylon or nitrocellulose) or a microsphere (bead) or a chip. Prior to application to the membrane or fixation it may be convenient to modify the nucleic acid probe in order to facilitate fixation or improve the hybridization efficiency. Such modifications may encompass homopolymer tailing, coupling with different reactive groups such as aliphatic groups, NH<sub>2</sub> groups, SH groups, carboxylic groups, or 20 coupling with biotin, haptens or proteins.

25 The term "labeled" refers to the use of labeled nucleic acids. Labeling may be carried out by the use of labeled nucleotides incorporated during the polymerase step of the amplification such as illustrated by Saiki et al. (1988) or Bej et al. (1990) or labeled primers, or by any other method known to the person skilled in the art. The nature of the label may be isotopic (<sup>32</sup>P, <sup>35</sup>S, etc.) or non-isotopic (biotin, digoxigenin, etc.).

30 The term "primer" refers to a single stranded oligonucleotide sequence capable of acting as a point of initiation for synthesis of a primer extension product, which is complementary to the nucleic acid strand to be copied. The length and the sequence of the primer must be such that they allow to prime the synthesis of the extension products. Preferably the primer is about 5-50 nucleotides long. Specific length and sequence will depend on the complexity of the required DNA or RNA targets, as well as on 35 the conditions of primer use such as temperature and ionic strength.

35 The term "primer pair" refers to a set of primers comprising at least one 5' primer and one 3' primer. The primer pair may consist of more than two primers, the complexity of the number of primers will depend on the hybridization conditions, variability of the sequences in the regions to be amplified and the target sequences to be detected.

The fact that amplification primers do not have to match exactly with the corresponding template

sequence to warrant proper amplification is amply documented in the literature (Kwok et al., 1990).

The amplification method used can be either polymerase chain reaction (PCR; Saiki et al., 1988), ligase chain reaction (LCR; Landgren et al., 1988; Wu & Wallace, 1989; Barany, 1991), nucleic acid sequence-based amplification (NASBA; Guatelli et al., 1990; Compton, 1991), transcription-based 5 amplification system (TAS; Kwoh et al., 1989), strand displacement amplification (SDA; Duck, 1990) or amplification by means of Q $\beta$  replicase (Lomeli et al., 1989) or any other suitable method to amplify nucleic acid molecules known in the art.

The oligonucleotides used as primers or probes may also comprise nucleotide analogues such 10 as phosphorothiates (Matsukura et al., 1987), alkylphosphorothiates (Miller et al., 1979) or peptide nucleic acids (Nielsen et al., 1991; Nielsen et al., 1993) or may contain intercalating agents (Asseline et al., 1984).

As most other variations or modifications introduced into the original DNA sequences of the invention these variations will necessitate adaptations with respect to the conditions under which the oligonucleotide should be used to obtain the required specificity and sensitivity. However the eventual 15 results of hybridization will be essentially the same as those obtained with the unmodified oligonucleotides.

The introduction of these modifications may be advantageous in order to positively influence characteristics such as hybridization kinetics, reversibility of the hybrid-formation, biological stability of the oligonucleotide molecules, etc.

20 The "sample" may be any biological material taken either directly from the infected human being (or animal), or after culturing (enrichment). Biological material may be e.g. expectorations of any kind, broncheolavages, blood, skin tissue, biopsies, sperm, lymphocyte blood culture material, colonies, liquid cultures, fecal samples, urine etc.

The sets of probes of the present invention will include at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 25 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30 or more probes. Said probes may be applied in two or more distinct and known positions on a solid substrate. Often it is preferable to apply two or more probes together in one and the same position of said solid support.

For designing probes with desired characteristics, the following useful guidelines known to the person skilled in the art can be applied.

30 Because the extent and specificity of hybridization reactions such as those described herein are affected by a number of factors, manipulation of one or more of those factors will determine the exact sensitivity and specificity of a particular probe, whether perfectly complementary to its target or not. The importance and effect of various assay conditions, explained further herein, are known to those skilled in the art.

35 The stability of the [probe : target] nucleic acid hybrid should be chosen to be compatible with the assay conditions. This may be accomplished by avoiding long AT-rich sequences, by terminating the

hybrids with G:C base pairs, and by designing the probe with an appropriate Tm. The beginning and end points of the probe should be chosen so that the length and %GC result in a Tm about 2-10°C higher than the temperature at which the final assay will be performed. The base composition of the probe is significant because G-C base pairs exhibit greater thermal stability as compared to A-T base pairs due  
5 to additional hydrogen bonding. Thus, hybridization involving complementary nucleic acids of higher G-C content will be stable at higher temperatures.

Conditions such as ionic strength and incubation temperature under which a probe will be used should also be taken into account when designing a probe. It is known that hybridization will increase as the ionic strength of the reaction mixture increases, and that the thermal stability of the hybrids will  
10 increase with increasing ionic strength. On the other hand, chemical reagents, such as formamide, urea, DMSO and alcohols, which disrupt hydrogen bonds, will increase the stringency of hybridization. Destabilization of the hydrogen bonds by such reagents can greatly reduce the Tm. In general, optimal hybridization for synthetic oligonucleotide probes of about 10-50 bases in length occurs approximately  
15 5°C below the melting temperature for a given duplex. Incubation at temperatures below the optimum may allow mismatched base sequences to hybridize and can therefore result in reduced specificity.

It is desirable to have probes, which hybridize only under conditions of high stringency. Under high stringency conditions only highly complementary nucleic acid hybrids will form; hybrids without a sufficient degree of complementarity will not form. Accordingly, the stringency of the assay conditions determines the amount of complementarity needed between two nucleic acid strands forming a hybrid.  
20 The degree of stringency is chosen such as to maximize the difference in stability between the hybrid formed with the target and the nontarget nucleic acid. In the present case, single base pair changes need to be detected, which requires conditions of very high stringency.

The length of the target nucleic acid sequence and, accordingly, the length of the probe sequence can also be important. In some cases, there may be several sequences from a particular region, varying  
25 in location and length, which will yield probes with the desired hybridization characteristics. In other cases, one sequence may be significantly better than another that differs merely by a single base. While it is possible for nucleic acids that are not perfectly complementary to hybridize, the longest stretch of perfectly complementary base sequence will normally primarily determine hybrid stability. While oligonucleotide probes of different lengths and base composition may be used, preferred oligonucleotide  
30 probes of this invention are between about 5 to 50 (more particularly 10-25) bases in length and have a sufficient stretch in the sequence which is perfectly complementary to the target nucleic acid sequence.

Regions in the target DNA or RNA, which are known to form strong internal structures inhibitory to hybridization, are less preferred. Likewise, probes with extensive self-complementarity should be avoided. As explained above, hybridization is the association of two single strands of complementary nucleic acids to form a hydrogen bonded double strand. It is implicit that if one of the  
35 two strands is wholly or partially involved in a hybrid that it will be less able to participate in formation

of a new hybrid. There can be intramolecular and intermolecular hybrids formed within the molecules of one type of probe if there is sufficient self complementarity. Such structures can be avoided through careful probe design. By designing a probe so that a substantial portion of the sequence of interest is single stranded, the rate and extent of hybridization may be greatly increased. Computer programs are 5 available to search for this type of interaction. However, in certain instances, it may not be possible to avoid this type of interaction.

Standard hybridization and wash conditions are disclosed in the Materials & Methods section of the Examples. Other conditions are for instance 3X SSC (Sodium Saline Citrate), 20% deionized FA (Formamide) at 50°C.

10 Other solutions (SSPE (Sodium saline phosphate EDTA), TMACl (Tetramethyl ammonium Chloride), etc.) and temperatures can also be used provided that the specificity and sensitivity of the probes is maintained. If need be, slight modifications of the probes in length or in sequence have to be carried out to maintain the specificity and sensitivity required under the given circumstances.

15 Primers may be labeled with a label of choice (e.g. biotin). Different primer-based target amplification systems may be used, and preferably PCR-amplification, as set out in the examples. Single-round or nested PCR may be used.

The term "hybridization buffer" means a buffer enabling a hybridization reaction to occur between the probes and the polynucleic acids present in the sample, or the amplified products, under the appropriate stringency conditions.

20 The term "wash solution" means a solution enabling washing of the hybrids formed under the appropriate stringency conditions.

The following examples only serve to illustrate the present invention. These examples are in no way intended to limit the scope of the present invention.

25 **FIGURE AND TABLE LEGENDS**

Figure 1: Natural and drug selected variability in the vicinity of codons 30, 46, 48, 50, 54, 82, 84, and 90 of the HIV-1 protease gene. The most frequently observed wild-type sequence is shown in the top line. Naturally occurring variations are indicated below and occur independently from each other.

30 Drug-selected variants are indicated in bold

Figure 2 A: Reactivities of the selected probes for codon 30 immobilized on LiPA strips with reference material. The information in the boxed surface is not relevant for the discussion of probes for condon 30. The position of each selected probe on the membrane strip is shown at the left of each panel. The 35 sequence of the relevant part of the selected probes is shown at the left and is given in Table 1. Each strip is incubated with a biotinylated PCR fragment from the reference panel. The reference panel accession

numbers are indicated in Table 1. For several probes multiple reference panel possibilities are available, but only one relevant accession number given each time. \*: False positive reactivities. At the bottom the strips, the amino acids at the relevant codon, as derived from the probe reactivity, is indicated.

5      Figure 2 B: Reactivities of the selected probes for codons 46 and 48 immobilized on LiPA strips with reference material. The information in the boxed surface is not relevant for the discussion of probes for condons 46 and 48. The position of each selected probe on the membrane strip is shown at the left of each panel. The sequence of the relevant part of the selected probes is given in Table 1. Each strip is incubated with a biotinylated PCR fragment from the reference panel. The reference panel accession numbers are  
10     indicated in Table 1. For several probes multiple reference panel possibilities are available, but only one relevant accession number given each time. \*: False positive reactivities. On top of the strips, the amino acids at the relevant codon, as derived from the probe reactivity, is indicated.

15     Figure 2 C: Reactivities of the selected probes for codon 50 immobilized on LiPA strips with reference material. The information in the boxed surface is not relevant for the discussion of probes for condon 50. The position of each selected probe on the membrane strip is shown at the left of each panel. The sequence of the relevant part of the selected probes is given in Table 1. Each strip is incubated with a biotinylated PCR fragment from the reference panel. The reference panel accession numbers are indicated in Table 1. For several probes multiple reference panel possibilities are available, but only one  
20     relevant accession number given each time. \*: False positive reactivities. At the bottom of the strips, the amino acids at the relevant codon, as derived from the probe reactivity, is indicated.

25     Figure 2 D: Reactivities of the selected probes for codon 54 immobilized on LiPA strips with reference material. The information in the boxed surface is not relevant for the discussion of probes for condon 54. The position of each selected probe on the membrane strip is shown at the left of each panel. The sequence of the relevant part of the selected probes is given in Table 1. Each strip is incubated with a biotinylated PCR fragment from the reference panel. The reference panel accession numbers are indicated in Table 1. For several probes multiple reference panel possibilities are available, but only one relevant accession number given each time. \*: False positive reactivities. At the bottom of the strips, the  
30     amino acids at the relevant codon, as derived from the probe reactivity, is indicated.

35     Figure 2 E.:Reactivities of the selected probes for codons 82 and 84 immobilized on LiPA strips with reference material. The information in the boxed surface is not relevant for the discussion of probes for condons 82 and 84. The position of each selected probe on the membrane strip is shown at the left of each panel. The sequence of the relevant part of the selected probes is given in Table 1. Each strip is incubated with a biotinylated PCR fragment from the reference panel. The reference panel accession

numbers are indicated in Table 1. For several probes multiple reference panel possibilities are available, but only one relevant accession number given each time. \*: False positive reactivities. At the bottom of the strips, the amino acids at the relevant codon, as derived from the probe reactivity, is indicated.

- 5     Figure 2 F: Reactivities of the selected probes for codon 90 immobilized on LiPA strips with reference material. The information in the boxed surface is not relevant for the discussion of probes for condon 90. The position of each selected probe on the membrane strip is shown at the left of each panel. The sequence of the relevant part of the selected probes is given in Table 1. Each strip is incubated with a biotinylated PCR fragment from the reference panel. The reference panel accession numbers are  
10    indicated in Table 1. For several probes multiple reference panel possibilities are available, but only one relevant accession number given each time. \*: False positive reactivities. At the bottom of the strips, the amino acids at the relevant codon, as derived from the probe reactivity, is indicated.

- 15    Figure 3: Sequence and position of the HIV-1 protease amplification primers. To obtain the reactivity with probes selected to determine the susceptibility to antiviral drugs involving codons 30, 46, 48, 50, 54, 82, and 84, nested amplification primers prot2bio(5' primer) and Prot31bio (3' primer) were designed. To obtain the reactivity with probes selected to determine the susceptibility to antiviral drugs involving codon 90, nested amplification primers Prot41bio (5' primer) and Prot6bio (3' primer) were designed.

- 20    Figure 4 A: Phylogenetic analysis on 312 protease sequences allowed to separate genotype B strains from non-B strains. Reactivities of the selected probes for codon 30 immobilized on LiPA strips with a biotinylated PCR fragment of genotype B strains and non-B strains is shown, the exact percentages are indicated in table 5. The probes are indicated at the bottom. The sequence of the relevant part of the probes is given in Table 1.  
25

- 30    Figure 4 B: Phylogenetic analysis on 312 protease sequences allowed to separate genotype B strains from non-B strains. Reactivities of the selected probes for codons 46/48 immobilized on LiPA strips with a biotinylated PCR fragment of genotype B strains and non-B strains is shown, the exact percentages are indicated in table 5. The probes are indicated at the bottom. The sequence of the relevant part of the probes is given in Table 1.

- 35    Figure 4 C: Phylogenetic analysis on 312 protease sequences allowed to separate genotype B strains from non-B strains. Reactivities of the selected probes for codon 50 immobilized on LiPA strips with a biotinylated PCR fragment of genotype B strains and non-B strains is shown, the exact percentages are indicated in table 5. The probes are indicated at the bottom. The sequence of the relevant part of the probes is given in Table 1.

Figure 4 D: Phylogenetic analysis on 312 protease sequences allowed to separate genotype B strains from non-B strains. Reactivities of the selected probes for codon 54 immobilized on LiPA strips with a biotinylated PCR fragment of genotype B strains and non-B strains is shown, the exact percentages are indicated in table 5. The probes are indicated at the bottom. The sequence of the relevant part of the probes is given in Table 1.

Figure 4 E: Phylogenetic analysis on 312 protease sequences allowed to separate genotype B strains from non-B strains. Reactivities of the selected probes for codons 82/84 immobilized on LiPA strips with a biotinylated PCR fragment of genotype B strains and non-B strains is shown, the exact percentages are indicated in table 5. The probes are indicated at the bottom. The sequence of the relevant part of the probes is given in Table 1.

Figure 4 F: Phylogenetic analysis on 312 protease sequences allowed to separate genotype B strains from non-B strains. Reactivities of the selected probes for codon 90 immobilized on LiPA strips with a biotinylated PCR fragment of genotype B strains and non-B strains is shown, the exact percentages are indicated in table 5. The probes are indicated at the bottom. The sequence of the relevant part of the probes is given in Table 1.

Figure 5 A: Geographical origin of 856 samples and reactivities with the different probes at codon position 30. The exact percentages are indicated in table 6. The probes are indicated at the bottom.

Figure 5 B: Geographical origin of 856 samples and reactivities with the different probes at codon positions 46/48. The exact percentages are indicated in table 6. The probes are indicated at the bottom.

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Figure 5 C: Geographical origin of 856 samples and reactivities with the different probes at codon position 50. The exact percentages are indicated in table 6. The probes are indicated at the bottom.

30

Figure 5 D: Geographical origin of 856 samples and reactivities with the different probes at codon position 54. The exact percentages are indicated in table 6. The probes are indicated at the bottom.

Figure 5 E: Geographical origin of 856 samples and reactivities with the different probes at codon positions 82/84. The exact percentages are indicated in table 6. The probes are indicated at the bottom.

35

Figure 5 F: Geographical origin of 856 samples and reactivities with the different probes at codon position 90. The exact percentages are indicated in table 6. The probes are indicated at the bottom.

Table 1: HIV-1 protease wild-type and drug-selected mutation probes with their corresponding sequences as applied on the HIV-1 protease LiPA strip. The most frequently observed wild-type sequence is shown at the top line. Probe names corresponding to the selected motifs are indicated in the left column, the relevant part of each probe applied on the strip is shown under the consensus sequence.

Table 2: Protease Inhibitors.

Table 3: HIV-1 protease wild-type and drug-selected mutation probes with their corresponding sequences as synthesized, immobilized and tested on LiPA strips. The most frequently observed wild-type sequence is shown at the top line. Probe names corresponding to the selected motifs are indicated in the left column, the relevant part of each probe applied on the strip is shown under the consensus sequence. The probes retained are indicated in table 1.

Table 4: Polymorphic nucleotide sequences.

Table 5: % Reactivities of the HIV-1 protease wild-type and drug-selected mutation probes applied on the HIV-1 protease LiPA strip with genotype B strains and non-B strains.

Table 6: % Reactivities of the HIV-1 protease wild-type and drug-selected mutation probes applied on the HIV-1 protease LiPA strip with samples of different geographical origin.

Table 7: HIV-1 protease wild-type and drug-selected mutation probes with their corresponding sequences as applied on the HIV-1 protease LiPA strip. The most frequently observed wild-type sequence is shown at the top line. Probe names corresponding to the selected motifs are indicated in the left column, the relevant part of each probe applied on the strip is shown under the consensus sequence.

## EXAMPLES

Example 1:

Selection of the plasma samples, PCR amplification and cloning of the PCR products.

Plasma samples (n=557) were taken from HIV type-1 infected patients and stored at -20°C until use. Plasma samples were obtained from naive and drug-treated patients. The drugs involved ritonavir, indinavir and saquinavir. The serum samples were collected from patients residing in Europe (Belgium, Luxembourg, France, Spain and UK), USA and Brazil.

HIV RNA was prepared from these samples using the guanidinium-phenol procedure. Fifty  $\mu$ l plasma was mixed with 150  $\mu$ l Trizol<sup>®</sup>LS Reagent (Life Technologies, Gent, Belgium) at room temperature (volume ratio: 1unit sample/ 3 units Trizol ). Lysis and denaturation occurred by carefully pipetting up and down several times, followed by an incubation step at room temperature for at least 5 minutes. Fourthy  $\mu$ l CHCl<sub>3</sub> was added and the mixture was shaken vigorously by hand for at least 15 seconds, and incubated for 15 minutes at room temperature. The samples were centrifuged at maximum 12,000g for 15 minutes at 4°C, and the colorless aqueous phase was collected and mixed with 100  $\mu$ l isopropanol. To visualize the minute amounts of viral RNA, 20  $\mu$ l of 1 $\mu$ g/ $\mu$ l Dextran T500 (Pharmacia) was added, mixed and left at room temperature for 10 minutes. Following centrifugation at max. 12,000g for 10 minutes at 4°C and aspiration of the supernatant, the RNA pellet was washed with 200  $\mu$ l ethanol, mixed by vortexing and collected by centrifugation at 7,500g for 5 minutes at 4°C. Finally the RNA pellet was briefly air-dried and stored at -20°C. Alternatively, the High Pure Viral Nucleic Acid Kit (Boehringer Mannheim ) was used to extract RNA from the samples

For cDNA synthesis and PCR amplification, the RNA pellet was dissolved in 15  $\mu$ l random primers (20 ng/ $\mu$ l, pdN<sub>6</sub>, Pharmacia), prepared in DEPC-treated or HPLC grade water. After denaturation at 70°C for 10 minutes, 5  $\mu$ l cDNA mix was added, composed of 4  $\mu$ l 5x AMV-RT buffer (250mM Tris.HCl pH 8.5, 100mM KCl, 30mM MgCl<sub>2</sub>, 25 mM DTT), 0.4  $\mu$ L 25mM dXTPs, 0.2  $\mu$ l or 25U Ribonuclease Inhibitor (HPRI, Amersham), and 0.3  $\mu$ l or 8U AMV-RT (Stratagene). cDNA synthesis occurred during the 90 minutes incubation at 42°C. The HIV -1 protease gene was than amplified using

the following reaction mixture: 5  $\mu$ l cDNA, 4.5  $\mu$ l 10x Taq buffer, 0.3  $\mu$ l 25 mM dXTPs, 1  $\mu$ l (10 pmol) of each PCR primer, 38  $\mu$ l H<sub>2</sub>O, and 0.2  $\mu$ l (1 U) Taq. . Alternatively, the Titon One Tube RT-PCR system (Boehringer Mannheim) was used to perform RT-PCR.

Codon positions involving resistance to saquinavir, ritonavir, indinavir, nelfinavir and VX-478 have been described (Shinazi *et al*) and PCR amplification primers were chosen outside these regions. The primer design was based on HIV-1 published sequences (mainly genotype B clade) (Myers *et al.*) and located in regions that showed a high degree of nucleotide conservation between the different HIV-1 clades. The final amplified region covered the HIV-1 protease gene from codon 9 to codon 99. The primers for amplification had the following sequence:

outer sense primer Pr16: 5' bio-CAGAGCCAACAGCCCCACCAG3' (SEQ ID NO 1); nested sense primer Prot 2 bio: 5' CCT CAR ATC ACT CTT TGG CAA CG 3' (SEQ ID NO 3) ; nested antisense primer Prot 6 bio: 3' TAA TCR GGA TAA CTY TGA CAT GGT C 5' (SEQ ID NO 4); and outer antisense primer RT12: 5' bioATCAGGATGGAGTTCATAACCCATCCA3' (SEQ ID NO 2). Annealing occurred at 57°C, extension at 72°C and denaturation at 94°C. Each step of the cycle took 1 minute, the outer PCR contained 40 cycles, the nested round 35. Nested round PCR products were analyzed on agarose gel and

only clearly visible amplification products were used in the LiPA procedure. Quantification of viral

RNA was obtained with the HIV Monitor<sup>TM</sup> test (Roche, Brussels, Belgium). Later on, new sets of primers for amplification were selected. For the amplification of HIV protease codon 30-84: outer sense primer prot16: 5'-CAGAGCCAACAGCCCCACCAG-3' (SEQ ID NO 501), outer antisense primer prot5: 5'-TTTCTTCTGTCAATGCCATTGTTT-3' (SEQ ID NO 502) were used. Annealing occurred at 50°C, extension at 68°C and denaturation at 94°C for 35 cycles for the outer PCR. For the nested PCR annealing occurred at 45°C, denaturation at 94°C and extension at 92°C with primers: nested sense primers prot2a-bio: 5'-bio-CCTCAAATCACTCTTGGCAACG-3' (SEQ ID NO 503) and prot2b-bio: 5'-bio-CCTCAGATCACTCTTGGCAACG-3' (SEQ ID NO 504), and nested antisense primer prot31-bio: 5'-bio-AGTCAACAGATTCTTCCAAT-3' (SEQ ID NO 6). For the amplification of HIV protease codon 90, the outer PCR was as specified for HIV protease codon 30-84. For the nested PCR, nested sense primer prot41-bio: 5'-bio-CCTGTCAACATAATTGCAAG-3' (SEQ ID NO 505) and nested antisense primers prot6a: 5'-bio-CTGGTACAGTTCAATAGGGCTAAT-3' (SEQ ID NO 506), prot6b: 5'-bio-CTGGTACAGTTCAATAGGACTAAT-3' (SEQ ID NO 507), prot6c: 5'-bio-CTGGTACAGTCTCAATAGGACTAAT-3' (SEQ ID NO 508), prot6d: 5'-bio-CTGGTACAGTCTCAATAGGGCTAAT-3' (SEQ ID NO 509) were used. For the nested PCR the annealing temperature occurred at 45°C. Primers were tested on a plasmid, which contained an HIV fragment of 1301 bp ligated in a pGEM-T vector. The fragment contains protease, reverse transcriptase and the primer sites of first and second round PCR. By restriction with *Sac I* the plasmid is linearised.

Selected PCR products were cloned into the pretreated EcoRV site of the pGEMT vector (Promega). Recombinant clones were selected after  $\alpha$ -complementation and restriction fragment length analysis, and sequenced using standard sequencing techniques with plasmid primers and internal HIV protease primers. Sometimes biotinylated fragments were directly sequenced with a dye-terminator protocol (Applied Biosystems) using the amplification primers. Alternatively, nested PCR was carried out with analogs of the nested primers, in which the biotin group was replaced with the T7- and SP6-primer sequence, respectively. These amplicons were then sequenced with an SP6- and T7-dye-primer procedure.

**Example 2:**

30   **Selection of a reference panel**

Codon positions involving resistance to saquinavir, ritonavir, indinavir, nelfinavir and VX-478 have been described (Shinazi *et al.* 1997). It was the aim to clone in plasmids those viral protease genes that are covering the different genetic motifs at those important codon positions conferring resistance against the described protease inhibitors.

35   After careful analysis of 312 protease gene sequences, obtained after direct sequencing of PCR fragments, a selection of 47 PCR fragments which covered the different target polymorphisms and

mutations were retained and cloned in plasmids using described cloning techniques. The selection of samples originated from naive or drug-treated European, Brazilian or US patients. These 47 recombinant plasmids are used as a reference panel, a panel that was sequenced on both strands, and biotinylated PCR products from this panel were used to optimize probes for specificity and sensitivity.

5 Although this panel of 47 samples is a representative selection of clones at this moment, it is important to mention here that this selection is in fact only a temporally picture of the variability of the virus, and a continuous update of this panel will be mandatory. This includes on ongoing screening for the new variants of the virus, and recombinant cloning of these new motifs.

10 **Probe selection and LiPA testing.**

To cover all the different genetic motifs in the reference panel, a total of 471 probes were designed (codon 30: 40 probes; codon 46/48: 72 probes; codon 50:55 probes; codon 54: 54 probes; codon 82/84: 130 probes; codon 90: 120 probes). Table 3 shows the different probes that were selected for the different codon positions.

15 It was the aim to adapt all probes to react specifically under the same hybridization and wash conditions by carefully considering the % (G+C), the probe length, the final concentration of the buffer components, and hybridization temperature (Stuyver et al., 1997). Therefore, probes were provided enzymatically with a poly-T-tail using the TdT (Pharmacia) in a standard reaction condition, and purified via precipitation. For a limited number of probes with 3' T-ending sequences, an additional G was  
20 incorporated between the probe sequence and the poly-T-tail in order to limit the hybridizing part to the specific probe sequence and to exclude hybridization with the tail sequence. Probe pellets were dissolved in standard saline citrate (SSC) buffer and applied as horizontal parallel lines on a membrane strip. Control lines for amplification (probe 5' TAGGGGAAATTGGAGGTTTAG 3', HIV protease aa 47 to aa 54) and conjugate incubation (biotinylated DNA) were applied alongside. Probes were  
25 immobilized onto membranes by baking, and the membranes were sliced into 4mm strips also called LiPA strips.

Selection of the amplification primers and PCR amplification was as described in example 1. In order to select specific reacting probes out of the 471 candidate probes, LiPA tests were performed with biotinylated PCR fragments from the reference panel. To perform LiPA tests, equal amounts (10  
30 µl) of biotinylated amplification products and denaturation mixture (0.4 N NaOH/0.1% SDS) were mixed, followed by an incubation at room temperature for 5 minutes. Following this denaturation step, 2 ml hybridization buffer (2xSSC, 0.1% SDS, 50mM Tris pH7.5) was added together with a membrane strip and hybridization was carried out at 39°C for 30 min. Then, the hybridization mixture was replaced by stringent washing buffer (same composition as hybridization buffer), and stringent washing occurred  
35 first at room temperature for 5 minutes and then at 39°C for another 25 minutes. Buffers were then replaced to be suitable for the streptavidine alkaline phosphatase conjugate incubations. After 30 minutes

incubation at room temperature, conjugate was rinsed away and replaced by the substrate components for alkaline phosphatase, Nitro-Blue-Tetrazolium and 5-Bromo-4-Chloro-3-Indolyl Phosphate. After 30 minutes incubation at room temperature, probes where hybridization occurred became visible because of the purple brown precipitate at these positions.

5        After careful analysis of the 471 probes, the most specific and sensitive probes (n=46) were finally selected, covering the natural and drug-selected variability in the vicinity of aa. 30, 46, 48, 50, 54, 82, 84, and 90. Figure 2 shows the reactivity of the finally selected probes with the reference panel.

**Example 3:**

10      **LiPA testing on clinical samples.**

A total of 856 samples were tested on this selection of 46 specific probes. The geographical origin of these samples is as follows: USA:359 ; France: 154; UK:36; Brazil 58; Spain 35; Belgium 199; Luxembourg: 15.

15      From this population, a total of 144 samples were sequenced which allowed to separate the genotype B samples (94) from the non-B samples (50). After analysis of these genotyped samples on LiPA, the genotypic reactivity on the selected probes was scored. Figures 4A to 4F show these results for the different codon positions and for the genotype B versus non-B group. From these tables, it is clear that there is little difference in sequence usage for the different codon positions with respect to specific reactivities at the different probes.

20      The total collection of 856 samples was then tested on the available 46 probes. After dissection of these reactivities over the different probes and different geographical origin, the picture looks as is presented in Figures 5A to 5F. Again here, the majority of the sequences used at the different codon positions are restricted to some very abundant wild type motifs. It is important to mention here that the majority of these samples are taken from patients never treated with protease inhibitors, en therefore, the 25 majority of the reactivities are found in wild type motifs. Nevertheless, it is clear from some codon positions that the variability at some codon positions in the mutant motif might be considerable, and again, a continuos update on heavily treated patients is mandatory. Another issue is the amount of double blank reactivities, which is in this approach reaching up to 5% in global; with some peak values for some countries for some codon positions: for example 13.8% for codon 82/85 in Brazil; and 18.1 % for codon 30 90 in Belgium.

The continuous update resulted in a further selection of probes. This later configuration of the strip is indicated in table 7.

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**Table 1**

	26	27	28	29	30	31	32	33	34	3	Tm	length	Seq	ID	
pc30w25	ACA	GGA	GCA	GAT	GAT	ACA	GTA	TTA	GAA	GAA					
			GCA	GAT	GAT	ACA	GT				40	14		31	
pc30w29		A	GCG	GAT	GAT	ACA						36	13		35
pc30w32			GCA	GAT	GAC	ACA	GT					42	14		38
pc30w36			GCA	GAC	GAT	ACA	GG					40	14		42
pc30m23		A	GCA	GAT	AAT	ACA	GT					40	15		29
	44	45	46	47	48	49	50	51	52						
pc48w47	CCA	AAA	ATG	ATA	GGG	GGA	ATT	GGA	GGT						
			AAA	ATG	ATA	GGG	GGA					42	15		93
pc48w45		A	ATG	ATA	GGA	GGA	ATT					42	16		91
pc48w72		A	AAA	ATA	ATA	GGG	GGA					42	16		120
pc48m41				ATG	ATA	GTG	GGA	ATT				40	15		87
	48	49	50	51	52	53	54								
pc50w31	GGG	GGA	ATT	GGA	GGT	TTT	ATC								
			GGA	ATT	GGA	GGT	TTT					42	15		151
pc50w44			GGA	ATT	GGG	GGT	TTG					42	15		164
pc50w52			GA	ATT	GGA	GTC	TTG						14		172
pc50m37	GGG	GGA	GTT	GGA								40	12		157
	51	52	53	54	55	56	57	58							
pc54w3	GGA	GGT	TTT	ATC	AAA	GTA	AGA	CAG							
			GT	TTT	ATC	AAA	GTA	AGA				42	17		178
pc54w34	GA	GGT	TTT	ATC	AAA	GT						42	16		212
pc54w14		GGT	TTT	ATC	AAG	GTA	A					42	16		189
pc54w19	A	GGC	TTT	ATC	AAA	GTA						42	16		194
pc54w22	GA	GGT	TTT	ATT	AAA	GTA						42	17		197
pc54w26	A	GGT	TTC	ATT	AAG	GTA						42	16		202
pc54w27		GGT	TTT	ATT	AAG	GTA	A					40	16		204
pc54m55	A	GGT	TTT	GCC	AAA	GT						38	15		
pc54m35		GGT	TTT	GTC	AAA	GTA						40	15		213
pc54m37		GGT	TTT	GTC	AGA	GTA						42	15		215
	78	79	80	81	82	83	84	85	86	87					
pc82w91	GGA	CCT	ACA	CCT	GTC	AAC	ATA	ATT	GGA	AGA					
			ACA	CCT	GTC	AAC	ATA	A			44	16			
pc82w60			CA	CCT	GTC	AAT	ATA	ATG			42	17		318	
pc82w111			A	CCG	GTC	AAC	ATA	ATT			44	16		287	
pc82w89			ACA	CCT	GTT	AAC	ATA	AG			42	17		338	
pc82w42			CA	CCT	GTC	AAC	GTA				42	14		316	
pc82m36			ACA	CCT	ACC	AAC	ATA				42	15		269	
pc82m67			ACA	CCT	ACC	AAC	GT				42	14		294	
pc82m38			ACA	CCT	TTC	AAC	ATA				40	15		265	
pc82m105			ACG	CCC	TTC	AAC	ATA				44	15		332	
pc82m127			CA	CCT	TTC	AAC	GTA	ATG			44	17		354	
pc82m40			ACA	CCT	GCC	AAC	ATA				44	15		267	
pc82m63			CA	CCT	GCC	AAT	ATA	AG			42	16		290	
pc82m101			ACA	CCT	ATC	AAC	ATA	ATG			44	18		328	

**Table 1 - Cont'd**

	86	87	88	89	90	91	92	93	94			
	GGA	AGA	AAT	CTG	TTG	ACT	CAG	ATT	GGT			
pc90w27			AAT	CTG	TTG	ACT	CA			38	14	384
pc90w37			AAT	CTG	TTG	ACT	CAG	ATG		42	18	394
pc90w39			GA	ACT	CTG	TTG	ACT	C		44	15	396
pc90w50			AAT	ATG	TTG	ACT	CAG			40	15	407
pc90w52			AAT	TTG	TTG	ACT	CAG			40	15	409
pc90w69			GA	AAC	CTG	TTG	ACT			40	14	426
pc90w73				TG	TTG	ACA	CAG	CTT	G	44	15	430
pc90w79				TG	TTG	ACC	CAG	ATT	G	44	15	436
pc90m43		A	AAT	CTG	ATG	ACT	CA			40	15	400
pc90m56			AAT	ATG	ATG	ACC	CAG			42	15	413

**Table 2**  
**Protease Inhibitors**

Compound	Amino acid change	Codon change
<b>Protease Inhibitors</b>		
A-77003	R8Q	CGA to CAA
	R8K	CGA to AAA
	V32I	GTA to ATA
	M46I	ATG to ATA
	M46L	ATG to TTC
	M46F	ATG to TTC
	M46V	ATG to GTG
	G48V	GGG to GTG
	A71V	GCT to GTT
	V82I	GTC to ATC
	V82A	GTC to GCC
	L63P	CTC to CCC
	A71T	GCT to ACT
	A71V	GCT to GTT
	G73S	GGT to GCT
	V82A	GTC to GCC
	V82F	GTC to TTC
	V82T	GTC to ACC
	I84V	ATA to GTA
	L90M	TTG to ATG
P9941	V82A	GTC to GCC
Ro 31-8959 (saquinavir)	L10I	CTC to ATC
	G48V	GGG to GTG
	I54V	ATC to GTC
	I54V	ATA to GTA
	G73S	GGT to AGT
	V82A	GTC to GCC
	I84V	ATA to GTA
	L90M	TTG to ATG
RPI-312	I84V	ATA to GTA

29  
**Table 2 - Cont'd-1**

SC-52151	L24V G48V A71V V75I P81T V82A N88D	TTA to GTA GGG to GTG GCT to GTT GTA to ATA CCT to ACT GTC to GCC AAT to GAT
SC-55389A	L10F N88S	CTC to CGC AAT to AGT
SKF108842	V82T I84V	GTC to ACC ATA to GTA
SKF108922	V82A V82T	GTC to GCC GTC to ACC
VB 11,328	L10F M46I I47V I50V I84V	CTC to GGC ATG to ATA ATA to CTA ATT to GTT ATA to GTA
VX-478 (141W94)	L10F M46I I47V I50V I84V	CTC to CGC ATG to ATA ATA to CTA ATT to GTT ATA to GTA
XM323	L10F K45I M46L V82A  V82I V82F I84V	CTC to CGC AAA to ATA ATG to CTG GTC to GCC  GTC to ATC GTC to TTC ATA to GTA
	L97V	TTA to GTA
	I82T	ATC to ACC
A-75925 ABT-538 (ritonavir)	V32I K20R L33F	GTA to ATA AAG to AAA TTA to TTC

Table 2 - Cont'd-2

	M36I M46I I54L I54V A71V V82F V82A V82T V82S I84V L90M	ATG to ATA ATG to ATA ATC to ? ATC to GTC GTC to GTT GTC to TTC GTC to GCC GTC to ACC GTC to TCC ATA to GTA TTG to ATG
AG1343 (nelfinavir)	D30N M36I M46I L63P A71V V77I I84V N88D L90M	GAT to AAT  ATG to ATA CTC to CCC GCT to GTT  ATA to GTA  TTG to ATG
BILA 1906 BS	V32I M46I M46L  A71V I84A I84V	GTA to ATA ATG to ATA ATG to TTG  GCT to GTT ATA to GCA ATA to GTA
BILA 2011 (palinavir)	V32I A71V I84A L63P	GTA to ATA GCT to GTT ATG to ATA CTC to CCC
BILA 2185 BS	L23I	CTA to ATA
BMS 186,318	A71T V82A	GCT to ACT GTC to GCC
DMP 450	L10F	CTC to TTC

**Table 2 - Cont'd-3**

M46I D60E I84V		ATG to ATA GAT to GAA ATA to GTA
KNI-272	V32I	GTA to ATA
MK-639 (L-735,524, indinavir)	L10I L10R L10V K20M K20R L24I V32I M46I M46L I54V	CTC to ATC CTC to CGC CTC to GTC AAG to ATG AAG to AAA TTA to ATA GTA to ATA ATG to ATA ATG to TTG ATC to GTC

Table 3

	26	27	28	29	30	31	32	33	34	35	length	Seq	ID
P30w1	ACA	GGA	GCA	GAT	GAT	ACA	GTA	TTA	GAA	GAA			
	A	GCA	GAT	GAT	ACA	GTA	TT				18		7
P30w2		GA	GCA	GAT	GAT	ACA	GTA	TT			19		8
P30w3		A	GCA	GAT	GAT	ACA	GTA	TTA			19		9
P30w4		GGA	GCA	GAT	GAT	ACA	GTA	TT			20		10
P30w5		GGA	GCA	GAT	GAT	ACA	GTA	TTA			21		11
P30w6	ACA	GGA	GCA	GAT	GAT	ACA					18		12
P30w7		CA	GGA	GCA	GAT	GAT	ACA	GT			19		13
P30w8		A	GGA	GCA	GAT	GAT	ACA	TG			20		14
P30w9			GGG	GCA	GAT	GAT	ACA	GTA	TG		19		15
P30w10	ACA	GGA	GCA	GAT	GAT	ACA	GG				19		16
P30m11			A	GCA	GAT	AAT	ACA	GTA	TT		18		17
P30m12			GA	GCA	GAT	AAT	ACA	GTA	TT		19		18
P30m13			A	GCA	GAT	AAT	ACA	GTA	TTA		19		19
P30m14			GGA	GCA	GAT	AAT	ACA	GTA	TT		20		20
P30m15			GGA	GCA	GAT	AAT	ACA	GTA	TTA		21		21
P30m15	ACA	GGA	GCA	GAT	AAT	ACA					18		22
P30m17		CA	GGA	GCA	GAT	AAT	ACA	GT			19		23
P30m18		A	GGA	GCA	GAT	AAT	ACA	GTA	TG		20		24
P30m19			GGA	GCA	GAT	AAT	ACA	GTA	TG		19		25
P30m20	ACA	GGA	GCA	GAT	AAT	ACA	GG				19		26
p30w21			A	GCA	GAT	GAT	ACA	GT			15		27
p30w22			A	GCA	GAT	GAT	ACA	GTA	G		16		28
p30m23			A	GCA	GAT	AAT	ACA	GTA			15		29
p30m24			A	GCA	GAT	AAT	ACA	GTA	G		16		30
p30w25				GCA	GAT	GAT	ACA	GT			14		31
p30w26			A	GCA	GAT	GAT	ACA	GG			14		32
p30w27				CA	GAT	GAT	ACA	GT			13		33
p30w28		GA	GCG	GAT	GAT	ACA					14		34
p30w29		A	GCG	GAT	GAT	ACA					13		35
p30m30			GCA	GAT	AAT	ACA	GTA				15		36
p30m31			GCA	GAT	AAT	ACA	GT				14		37
p30w32			GCA	GAT	GAC	ACA	GT				14		38
p30w33				CA	GAT	GAC	ACA	GTA	G		14		39
p30w34				CA	GAT	GAT	ACA	ATA	TT		16		40
p30w35				GCA	GAT	GAT	ACA	ATA	TG		16		41
p30w36				GCA	GAC	GAT	ACA	GG			13		42
p30w37				GCA	GAC	GAT	ACA	GT			14		43
p30w38				A	GAT	GAT	ACA	ATA	TT		15		44
p30w39				A	GAT	GAT	ACA	ATA	TTA		16		45
p30w40				GCA	GAT	GAT	ACA	ATA			15		46

Table 3 - Cont'd-1

	44	45	46	47	48	49	50	51	52	53	54	length	Seq	ID
	CCA	AAA	ATG	ATA	GGG	GGA	ATT	GGA	GGT	TTT	ATC			
P48w1				GTA	GGG	GGA	ATT	GGA	GGT	GG		18		47
P48w2				GTA	GGG	GGA	ATT	GGA	GGT	TG		19		48
P48w3				GTA	GGG	GGA	ATT	GGA	GGT	TTG		20		49
P48w4				GTA	GGG	GGA	ATT	GGA	GGT	TTT		21		50
P48w5			G	GTA	GGG	GGA	ATT	GGA	GGT	TTG		21		51
P48w6			ATG	GTA	GGG	GGA	ATT	GGA				18		52
P48w7			ATG	GTA	GGG	GGA	ATT	GGA	G			19		53
P48w8		A	ATG	GTA	GGG	GGA	ATT	GGA				19		54
P48w9		A	ATG	GTA	GGG	GGA	ATT	GGA	G			20		55
P48w10		A	ATG	GTA	GGG	GGA	ATT	GGA	GGG	GG		22		56
P48w21			ATA	ATA	GGG	GGA	ATT	GGA				18		57
P48w22			ATG	ATA	GGG	GGA	ATT	GGA				18		58
P48w23		A	ATA	ATA	GGG	GGA	ATT	GGA				19		59
P48w24		A	ATG	ATA	GGG	GGA	ATT	GGA				19		60
P48w25				ATA	GGG	GGA	ATT	GGA	GGT	GG		18		61
P48w26				ATA	GGG	GGA	ATT	GGA	GGT	TG		19		62
P48w28				ATA	GGG	GGA	ATT	GGA	GGT	TTG		20		63
P48w29				ATA	GGG	GGA	ATT	GGA	GGT	TTT		21		64
P48m11				GTA	GTG	GGA	ATT	GGA	GGT	GG		18		65
P48m12				GTA	GTG	GGA	ATT	GGA	GGT	TG		19		66
P48m13				GTA	GTG	GGA	ATT	GGA	GGT	TTG		20		67
P48m14				GTA	GTG	GGA	ATT	GGA	GGT	TTT		21		68
P48m15		G	GTA	GTG	GGA	ATT	GGA	GGT	TTG			21		69
P48m16			ATG	GTA	GTG	GGA	ATT	GGA				18		70
P48m17			ATG	GTA	GTG	GGA	ATT	GGA	G			19		71
P48m18		A	ATG	GTA	GTG	GGA	ATT	GGA				19		72
P48m19		A	ATG	GTA	GTG	GGA	ATT	GGA	G			20		73
P48m20		A	ATG	GTA	GTG	GGA	ATT	GGA	GGG	GG		22		74
P48m29				ATA	GTG	GGA	ATT	GGA	GGT	GG		18		75
P48m30				ATA	GTG	GGA	ATT	GGA	GGT	TG		19		76
P48m31				ATG	ATA	GTG	GGA	ATT	GGA			18		77
P48m32				ATG	ATA	GTG	GGA	ATT	GGA	G		19		78
P48m33		A	ATG	ATA	GTG	GGA	ATT	GGA				19		
p48w34			G	ATA	GGG	GGA	ATT	G				19		79
p48w35				TG	ATA	GGG	GGA	ATT	G			14		80
p48w36				TG	ATA	GGG	GGA	ATT	GG			15		81
p48w37				ATG	ATA	GGG	GGA	ATT				16		82
p48m38				G	ATA	GTG	GGA	ATT				15		83
p48m39				TG	ATA	GTG	GGA	ATT	G			14		84
p48m40				TG	ATA	GTG	GGA	ATT	GG			15		85
p48m41				ATG	ATA	GTG	GGA	ATT				16		86
p48w42				ATA	ATA	GGG	GGA	ATT				15		87
p48w43					TG	ATA	GGG	GGA	GTT			15		88
p48w44					G	ATA	GGG	GGA	GTT	G		14		89
p48w45		A	ATG	ATA	GGA	GGA	ATT					14		90
p48w46					ATG	ATA	GGG	GGA	ATT			16		91
p48w47					AAA	ATG	ATA	GGG	GGA			15		92
p48w48					A	AAA	ATG	ATA	GGG	GG		15		93
												15		94

**Table 3 - Cont'd-2**

p48w49	AA	ATG	ATA	GGG	GGA	AG	15	95
p48w50	AAA	ATA	ATA	GGG	GGA	AG	16	96
p48w51	AAA	ATA	AAA	AT			15	97
p48m52	AAA	ATG	ATA	GTG	GGA	AG	16	98
p48w52b	AAA	TTG	ATA	GGG	GG		14	99
p48m53	AAA	ATG	ATA	GTG	GGA		15	100
p48w53b	AAA	TTG	ATA	GGG	GGA		15	101
p48w54	CA	AAA	TTG	ATA	G		15	102
p48w55			ATG	GTA	GGG	GGA ATT	15	103
p48w56	AA	ATG	GTA	GGG	GGA		14	104
p48w57	A	AAA	ATG	GTA	GGG	G	14	105
p48w58			ATG	ATA	GGG	GAA ATT	15	106
p48w59				ATA	GGG	GAA ATT GGA	15	107
p48w60				ATA	GGG	GAA ATT GGA G	16	108
p48w61			ATG	ATA	GGG	GGG ATT	15	109
p48w62				ATA	GGG	GGG ATT GG	14	110
p48w63				A	GGG	GGG ATT GGA	13	111
p48m64	AAA	ATA	ATA	GTG	GGA		15	112
p48m65	A	AAA	ATA	ATA	GTG	GGA	16	113
p48m66	CA	AAA	ATA	ATA	GTG	GG	16	114
p48m67		AAA	TTG	ATA	GTG	GGA	15	115
p48m68	A	AAA	TTG	ATA	GTG	GGA	16	116
p48m69	CA	AAA	TTG	ATA	GTG	G	15	117
p48w70			AAA	ATG	ATA	GGG GG	14	118
p48w71	A	AAA	ATG	ATA	GGG	G	14	119
pc48w72	A	AAA	ATA	ATA	GGG	GGA	16	120

**Table 3 - Cont'd-3**

	45	46	47	48	49	50	51	52	53	54	length	Seq	ID		
	AAA	ATG	GTA	GGG	GGA	ATT	GGA	GGT	TTT	ATC					
P50w1				GGG	GGA	ATT	GGA	GGT	TTT		18		121		
P50w2				A	GGG	GGA	ATT	GGA	GGT	TTT		19		122	
P50w3				TA	GGG	GGA	ATT	GGA	GGT	TTT		20		123	
P50w4				A	GGG	GGA	ATT	GGA	GGT	TTT	AG	20		124	
P50w5				TA	GGG	GGA	ATT	GGA	GGT	TTT	AG	21		125	
P50w6				GTA	GGG	GGA	ATT	GGA	GGT	TGG		19		126	
P50w7				G	GTA	GGG	GGA	ATT	GGA	GGT	TGG		20		127
P50w8					GTA	GGG	GGA	ATT	GGA	GGT	TTG		20		128
P50w9					GTA	GGG	GGA	ATT	GGA	GGT	TTT		20		129
P50w10			TG	GTA	GGG	GGA	ATT	GGA	GGT	GG		20		130	
p50w21					GG	GGA	ATT	GGA	GGT	TTT		17		131	
P50w22					GG	GGA	ATT	GGA	GGT	TTG		16		132	
P50w23					GG	GGA	ATT	GGA	GGT	TTT	AG	18		133	
P50w24					GG	GGA	ATT	GGA	GGT	TG		15		134	
P50w25					G	GGA	ATT	GGA	GGT	TTT	AT	18		135	
P50w26					GG	GGA	ATT	GGA	GGT	TTT		17		136	
P50m11					GGG	GGA	GTT	GGA	GGT	TTT		18		137	
P50m12				A	GGG	GGA	GTT	GGA	GGT	TTT		19		138	
P50m13				TA	GGG	GGA	GTT	GGA	GGT	TTT		20		139	
P50m14				A	GGG	GGA	GTT	GGA	GGT	TTT	AG	20		140	
P50m15				TA	GGG	GGA	GTT	GGA	GGT	TTT	AG	21		141	
P50m16				GTA	GGG	GGA	GTT	GGA	GGT	TGG		19		142	
P50m17			G	GTA	GGG	GGA	GTT	GGA	GGT	TGG		20		143	
P50m18					GTA	GGG	GGA	GTT	GGA	GGT	TTG		20		144
P50m19					GTA	GGG	GGA	GTT	GGA	GGT	TTT	ATC	21		145
P50m20			TG	GTA	GGG	GGA	GTT	GGA	GGT	GG		20		146	
P50m27					GG	GGA	GTT	GGA	GGT	TTG		19		147	
P50m28					GG	GGA	GTT	GGA	GGT	TTT	AG	18		148	
P50m29					GG	GGA	GTT	GGA	GGT	TG		15		149	
P50m30					G	GGA	GTT	GGA	GGT	TTT	AT	18		150	
p50w31					GGA	ATT	GGA	GGT	TTT			15		151	
p50w32				G	GGA	ATT	GGA	GGT	TGG			15		152	
p50m33					GGA	GTT	GGA	GGT	TTT			15		153	
p50m34				G	GGA	GTT	GGA	GGT	TGG			14		154	
p50m35				GGG	GGA	GTT	GGA	G				13		155	
p50m36				GG	GGA	GTT	GGA	G				12		156	
p50m37				GGG	GGA	GTT	GGA					12		157	
p50w38					GGA	ATT	GGG	GGT	TTG			14		158	
p50w39					GA	ATT	GGG	GGT	TTT			14		159	

**Table 3 - Cont'd-4**

p50w40	GA ATT GGG GGT TTT AG	15	160
p50w41	GGA ATT GGG GGT TG	13	161
p50w42	GGA ATT GGG GGT G	12	162
p50w43	GA ATT GGG GGT TG	12	163
p50w44	GA ATT GGG GGT TTG	13	164
p50w45	GGG GGA ATT GCA G	13	165
p50w46	GGA ATT GCA GGT TG	14	166
p50w47	GGA ATT GCA GGT G	13	167
p50w48	GGA ATT GGA GGG TTG	14	168
p50w49	GA ATT GGA GGG TTG	13	169
p50w50	GA ATT GGA GGG TTT	14	170
p50w51	GGA ATT GGA GGC TTG	14	171
p50w52	GA ATT GGA GGC TTG	13	172
p50w53	GA ATT GGA GGC TTT	14	173
p50m54	GGA GTT GGA GGT TTG	15	174
p50m55	GA GTT GGA GGT TTT	14	175

Table 3 - Cont'd-5

	51	52	53	54	55	56	57	58	length	Seq	ID
	GGA	GGT	TTT	ATC	AAA	GTA	AGA	CAG			
p54w1		GGT	TTT	ATC	AAA	GTA	A		16	176	
p54w2			GT	TTT	ATC	AAA	GTA	AG	16	177	
p54w3			GT	TTT	ATC	AAA	GTA	AGA	17	178	
p54w4			T	TTT	ATC	AAA	GTA	AGA	16	179	
p54w5		GGT	TTT	ATC	AAA	GTA			15	180	
p54w6			GT	TTT	ATC	AAA	GTA		15	181	
p54m7		GGT	TTT	GCC	AAA	GTA			15	182	
p54m8			GT	TTT	GCC	AAA	GTA	A	15	183	
p54m9			GT	TTT	GCC	AAA	GTA	AG	16	184	
p54m10			T	TTT	GCC	AAA	GTA	AGA	16	185	
p54m11		GGT	TTT	GCC	AAA	GT			14	186	
p54m12			GT	TTT	GCC	AAA	GTA		14	187	
p54w13			GT	TTT	ATC	AAG	GTA	AA	16	188	
p54w14		GGT	TTT	ATC	AAG	GTA	A		16	189	
p54w15		A	GGT	TTT	ATC	AAG	GTA		16	190	
p54w16			GT	TTT	ATC	AAA	GTC	AGA	17	191	
p54w17				TTT	ATC	AAA	GTC	AGA C	16	192	
p54w18		A	GGC	TTT	ATC	AAA	GTA	A	17	193	
p54w19		A	GGC	TTT	ATC	AAA	GTA		16	194	
p54m20		A	GGT	TTT	ATT	AAA	GTA	A	17	195	
p54m21			GGT	TTT	ATT	AAA	GTA	AG	17	196	
p54w22		GA	GGT	TTT	ATT	AAA	GTA		17	197	
p54m22		GA	GGT	TTT	ATT	AAA	GTA		17	198	
p54m23			GGT	TTT	ATT	GGT	TTT	AT	16	199	
p54m24			GGT	TTC	ATT	AAG	GTA		15	200	
p54m25			GGT	TTC	ATT	AAG	GTA	A	16	201	
p54w26		A	GGT	TTC	ATT	AAG	GTA		16	202	
p54m26		A	GGT	TTC	ATT	AAG	GTA		16	203	
p54w27			GGT	TTT	ATT	AAG	GTA	A	16	204	
p54m27			GGT	TTT	ATT	AAG	GTA	A	16	205	
p54m28		A	GGT	TTT	ATT	AAG	GTA		16	206	
p54m29		GA	GGT	TTT	ATT	AAG	GT		16	207	
p54m30			GGT	TTT	ATT	AAG	GTA	AG	17	208	
p54w31			GGT	TTT	ATC	AAA	GTA	A	16	209	
p54w32		A	GGT	TTT	ATC	AAA	GTA	A	17	210	
p54w33		A	GGT	TTT	ATC	AAA	GTA		16	211	
p54w34		GA	GGT	TTT	ATC	AAA	GT		16	212	
p54m35			GGT	TTT	GTC	AAA	GTA		15	213	
p54m36			GGT	TTT	GTC	AAA	GTA	A	16	214	
p54m37			GGT	TTT	GTC	AGA	GTA		15	215	
p54m38			GGT	TTT	GTC	AGA	GTA	A	16	216	
p54w39			GGG	TTT	ATC	AAA	GTA		15	217	
p54w40			GGG	TTT	ATC	AAA	GTA	A	16	218	
p54w41			GGC	TTC	ATC	AAA	GT		14	219	
p54w42		GA	GGC	TTC	ATC	AAA			14	220	
p54m48			GGT	TTT	GTC	AAA	GT		14	221	
p54m49			GT	TTT	GTC	AGA	GTA		14	222	

**Table 3 - Cont'd-6**

p54m50	GGT	TTT	GTC	AGA	GT	14	223	
p54w51	A	GGT	TTA	ATC	AAA	GTA	16	224
p54w52	GA	GGT	TTA	ATC	AAA	GT	16	225
p54m53		GGT	TTT	ACC	AAA	GTA	15	226
p54m54		GGT	TTT	ACC	AAA	GT	14	227

Table 3 - Cont'd-7

	78	79	80	81	82	83	84	85	86	87	length	Seq	ID
GGA	CCT	ACA	CCT	GTC	AAC	ATA	ATT	GGA	AGA				
P82w1	CCT	ACA	CCT	GTC	AAC	ATA	AG			19	228		
P82w2	CCT	ACA	CCT	GTC	AAC	ATA	ATG			20	229		
P82w3	CCT	ACA	CCT	GTC	AAC	ATA	ATT			21	230		
P82w4	A	CCT	ACA	CCT	GTC	AAC	ATA	AG		20	231		
P82w5	A	CCT	ACA	CCT	GTC	AAC	ATA	ATG		21	232		
P82w6	A	CCT	ACA	CCT	GTC	AAC	ATA			19	233		
P82w7	GA	CCT	ACA	CCT	GTC	AAC	ATA			20	234		
P82w8		CA	CCT	GTC	AAC	ATA	ATT	GGA		20	235		
P82w9		A	CCT	GTC	AAC	ATA	ATT	GGA	A	20	236		
P82w10		ACA	CCT	GTC	AAC	ATA	ATT	GG		20	237		
P82W21		A	CCT	GTC	AAC	ATA	ATT	GGA		19	238		
P82m11	CCT	ACA	CCT	ACC	AAC	ATA	AG			19	239		
P82m12	CCT	ACA	CCT	ACC	AAC	ATA	ATG			20	240		
P82m13	CCT	ACA	CCT	ACC	AAC	ATA	ATT			21	241		
P82m14	A	CCT	ACA	CCT	ACC	AAC	ATA	AG		20	242		
P82m15	A	CCT	ACA	CCT	ACC	AAC	ATA	ATG		21	243		
P82m16	A	CCT	ACA	CCT	ACC	AAC	ATA			19	244		
P82m17	GA	CCT	ACA	CCT	ACC	AAC	ATA			20	245		
P82m18		CA	CCT	ACC	AAC	ATA	ATT	GGA		20	246		
P82m19		A	CCT	ACC	AAC	ATA	ATT	GGA	A	20	247		
P82m20		ACA	CCT	ACC	AAC	ATA	ATT	G		19	248		
P82m22	CCT	ACA	CCT	TTC	AAC	ATA	ATT			21	249		
P82m23	CCT	ACA	CCT	GCC	AAC	ATA	ATT			21	250		
P82m24	CCT	ACA	CCT	TCC	AAC	ATA	ATT			21	251		
P82m25		A	CCT	TTC	AAC	ATA	ATT	GGA	A	20	252		
P82m26		A	CCT	GCC	AAC	ATA	ATT	GGA	A	20	253		
P82m27		A	CCT	TTC	AAC	ATA	ATT	GGA	A	20	254		
P82m28		A	CCT	ACC	AAC	ATA	ATT			16	255		
P82m29		A	CCT	TTC	AAC	ATA	ATT			19	256		
P82m30		A	CCT	GCC	AAC	ATA	ATT	GGA		19	257		
P82m31		A	CCT	TCC	AAC	ATA	ATT	GGA		19	258		
P82w32	T	ACA	CCT	GTC	AAC	AT				15	259		
P82w33	T	ACA	CCT	GTC	AAC	ATA				16	260		
P82w34		ACA	CCT	GTC	AAC	ATA				15	261		
P82w35		CA	CCT	GTC	AAC	ATA				14	262		
P82m36	ACA	CCT	ACC	AAC	ATA					15	263		
P82m37	CA	CCT	ACC	AAC	ATA					14	264		
P82m38	ACA	CCT	TTC	AAC	ATA					15	265		
P82m39	CA	CCT	TTC	AAC	ATA					14	266		
P82m40	ACA	CCT	GCC	AAC	ATA					15	267		
P82m41	CA	CCT	GCC	AAC	ATA					14	268		
P82w42	CA	CCT	GTC	AAC	GTA					14	269		
P82w43	CA	CCT	GTC	AAC	GT					13	270		
P82w44	CCT	ACA	CCT	GTC	AAC					15	271		
P82w45	T	ACG	CCT	GTC	AAC	AT				15	272		
P82w46	CT	ACG	CCT	GTC	AAC	AG				15	273		
P82m47	ACA	CCT	TCC	AAC	ATA					15	274		

Table 3 - Cont'd-8

P82m48	CA CCT TCC AAC ATA	14	275
P82m49	ACA CCT TCC AAC AT	14	276
P82m50	ACA CCT ATC AAC ATA	15	277
P82m51	CA CCT ATC AAC ATA AG	15	278
P82m52	CA CCT ATC AAC ATA ATG	16	279
P82m53	A CCT ATC AAC ATA ATG	15	280
P82w54	CCT GTC AAC ATA ATT	15	281
P82w55	CCT GTT AAC ATA ATT G	16	282
P82w56	A CCT GTT AAC ATA ATG	15	283
P82w57	CCG GTC AAC ATA ATT	15	284
P82w58	ACG CCT GTC AAC AT	14	285
P82w59	CCT GTC AAT ATA ATT	15	286
P82w60	CA CCT GTC AAT ATA ATG	16	287
P82w61	ACA CCT GTC AAT ATA AG	16	288
P82m62	CCT GCC AAT ATA ATT	15	289
P82m63	CA CCT GCC AAT ATA AG	15	290
P82m64	CCT ACC AAC GTA ATT	15	291
P82m65	CCT ACC AAC GTA ATG	14	292
P82m66	CA CCT ACC AAC GTA	14	293
P82m67	ACA CCT ACC AAC GT	14	294
P82m68	CCT TTC AAC GTA ATT	15	295
P82m69	CA CCT TTC AAC GTA AG	15	296
P82m70	ACA CCT TTC AAC GTA	15	297
P82m71	A CCT TTC AAC GTA ATG	15	298
p82w72	CT GTC AAT ATA ATT G	15	299
p82w73	CCT GTC AAT ATA ATT G	16	300
p82w74	A CCT GTC AAT ATA ATT	16	301
p82w75	CT GTC AAT ATA ATT GG	16	302
p82w76	CCT ACG CCT GTC AA	14	303
p82w77	CT ACG CCT GTC AAC	14	304
p82w78	A CCT ACG CCT GTC AA	15	305
p82w79	A CCT ACG CCT GTC A	14	306
p82w80	T ACA CCG GTC AAC A	14	307
p82w81	CT ACA CCG GTC AA	13	308
p82w82	CCT ACA CCG GTC A	13	309
p82w83	CA CCT GTC AAC ATA A	15	310
p82w84	A CCT GTC AAC ATA AT	15	311
p82w85	CT ACA CCT GTC AAC A	15	312
p82w86	ACA CCT GTC AAC AT	14	313
p82w87	A CCT GTT AAC ATA ATT G	17	314
p82w88	CA CCT GTT AAC ATA AG	15	315
p82w89	ACA CCT GTT AAC ATA AG	16	316
p82w90	TCA CCT GTC AAC ATA	14	317
p82w91	ACA CCT GTC AAC ATA A	16	318
p82w92	CA CCT GTC AAC ATA AT	16	319
p82w93	CCT GTC AAC ATA ATT	15	320
p82w94	A CCT GTC AAC ATA ATT	16	321
p82w95	CCT GTC AAC ATA ATT G	16	322
P82w96	CCT ACA CCT GTC AA	14	323
p82w97	T GTC AAC ATA ATT GG	15	324
p82w98	T GTC AAC ATA ATT GGA	16	325

**Table 3 - Cont'd-9**

p82m99	A	ACA	CCT	TTC	AAC	ATA	A	16	326
p82m100	T	ACA	CCT	TTC	AAC	ATA		16	327
p82m101		ACA	CCT	ATC	AAC	ATA	ATG	17	328
P82m102		ACA	CCT	ATC	AAC	ATA	AG	16	329
p82m103		CA	CCT	GCC	AAT	ATA	ATG	16	330
p82m104		ACA	CCT	GCC	AAT	ATA	AG	16	331
p82m105		ACG	CCC	TTC	AAC	ATA		15	332
p82m106		CG	CCC	TTC	AAC	ATA	AG	15	333
p82m107	T	ACG	CCC	TTC	AAC	AT		15	334
p82w108	CT	ACA	CCG	GTC	AAC			14	335
p82w109	CCT	ACA	CCG	GTC	AA			14	336
p82w110		A	CCG	GTC	AAC	ATA	ATG	15	337
p82w111		A	CCG	GTC	AAC	ATA	ATT	16	338
p82w112	CT	ACA	CCA	GTC	AAC			14	339
p82w113	CT	ACA	CCA	GTC	AAC	A		15	340
p82w114		ACA	CCA	GTC	AAC	ATA		15	341
p82w115		ACA	CCA	GTC	AAC	ATA	AG	16	342
p82w116	T	ACG	CCT	GTC	AAC	AT		15	343
p82w117		ACG	CCT	GTC	AAC	ATA		15	344
p82w118	T	ACG	CCT	GTC	AAC	A		14	345
p82m119	CCT	ACA	CCT	TTC	AAC			15	346
p82m120	CT	ACA	CCT	TTC	AAC			14	347
p82m121	A	CCT	ACA	CCT	TTC	AA		15	348
p82w122		ACG	CCT	GTC	AAC	ATA	AGG	16	349
p82w123	T	ACG	CCT	GTC	AAC	ATA		16	350
p82w124		CG	CCT	GTC	AAC	ATA	AGG	15	351
p82m125	T	ACA	CCT	TTC	AAC	GTA		16	352
p82m126		ACA	CCT	TTC	AAC	GTA	AGG	16	353
p82m127		CA	CCT	TTC	AAC	GTA	ATG	16	354
p82m128		A	CCT	TTC	AAC	GTA	ATT	16	355
p82o129			C	AAC	GTA	ATT	GGA AGA	16	356
p82o130			C	AAC	GTA	ATT	GGA AG	15	357

**Table 3 - Cont'd-10**

	86	87	88	89	90	91	92	93	94	length	Seq	ID	
P90w1	GGA	AGA	AAT	CTG	TTG	ACT	CAG	ATT	GGT				
		A	AAT	CTG	TTG	ACT	CAG			16	358		
P90w2		GA	AAT	CTG	TTG	ACT	CAG			17	359		
P90w3		GA	AAT	CTG	TTG	ACT	CAG	AGG		18	360		
P90w4		A	AAT	CTG	TTG	ACT	CAG	AGG		17	361		
P90w5		AGA	AAT	CTG	TTG	ACT	CAG	AGG		19	362		
P90w6		AGA	AAT	CTG	TTG	ACT	CAG	ATG		20	363		
P90w7		AGA	AAT	CTG	TTG	ACT	CAG	ATT		21	364		
P90w8		AGA	AAT	CTG	TTG	ACT	CAG	ATT		20	365		
P90w9		GA	AGA	AAT	CTG	TTG	ACT	CAG	AGG	21	366		
P90w10		A	AGA	AAT	CTG	TTG	ACT	CAG	ATG	21	367		
P90m11		AGA	AAT	CTG	ATG	ACT	CAG	ATG		20	368		
P90m12		AGA	AAT	CTG	ATG	ACT	CAG	ATT		21	369		
P90m13		A	AGA	AAT	CTG	ATG	ACT	CAG	AGG	20	370		
P90m14		GA	AGA	AAT	CTG	ATG	ACT	CAG	AGG	21	371		
P90m15		A	AGA	AAT	CTG	ATG	ACT	CAG	ATG	21	372		
P90m16		GA	AGA	AAT	CTG	ATG	ACT	CAG	ATT	20	373		
P90m17		GGA	AGA	AAT	CTG	ATG	ACT	CAG		21	374		
P90m18		A	AGA	AAT	CTG	ATG	ACT	CAG		19	375		
P90m19		A	AAT	CTG	ATG	ACT	CAG	ATT	GG	21	376		
P90m20		A	AAT	CTG	ATG	ACT	CAG	ATT	G	20	377		
P90m21		A	AAT	CTG	ATG	ACT	CAG	CTT	G	20	378		
P90m22		A	AAT	CTG	ATG	ACT	CAG	CTT		19	379		
P90m23			AAT	CTG	ATG	ACT	CAG	CTT	G	18	380		
P90w24		A	AAT	CTG	TTG	ACT	CAG	CTT	G	20	381		
P90w25		A	AAT	CTG	TTG	ACT	CAG	CTT		19	382		
P90w26			AAT	CTG	TTG	ACT	CAG	CTT	G	19	383		
P90w27			AAT	CTG	TTG	ACT	CA			14	384		
P90w28			AAT	CTG	TTG	ACT	CAG			15	385		
P90w29		A	AAT	CTG	TTG	ACT	CA			15	386		
P90w30		A	AAT	CTG	TTG	ACT	CAG			16	387		
P90m31			AAT	CTG	ATG	ACT	CA			14	388		
P90m32			AAT	CTG	ATG	ACT	CAG			15	389		
P90m33		A	AAT	CTG	ATG	ACT	CA			15	390		
P90m34		A	AAT	CTG	ATG	ACT	CAG			16	391		
P90w35		GA	AAT	CTG	TTG	ACT	C			15	392		
P90w36		GA	ACT	CTG	TTG	ACT	C			15	393		
P90w37			T	CTG	TTG	ACT	CAG	ATG		15	394		
P90w38		GA	AAT	CTG	TTG	ACT	C			15	395		
P90w39		GA	ACT	CTG	TTG	ACT	C			15	396		
P90w40		A	AAT	CTG	TTG	ACT	CA			15	397		
P90w41			AAT	CTG	TTG	ACT	CAG			15	398		
P90m42			AAT	CTG	ATG	ACT	CAG			15	399		
P90m43		A	AAT	CTG	ATG	ACT	CA			15	400		
P90w44				AT	CTG	TTG	ACT	CAG	AG	15	401		
P90w45					CTG	TTG	ACT	CAG	ATT	15	402		
P90w46		AGA	AAT	CTG	TTG	ACT				15	403		
P90m47					AT	CTG	ATG	ACT	CAG	AG	15	404	

**Table 3 - Cont'd-11**

P90m48	CTG	ATG	ACT	CAG	ATT	15	405	
P90m49	AGA	AAT	CTG	ATG	ACT	CA	17	406
P90w50	AAT	ATG	TTG	ACT	CAG	15	407	
P90w51	GA	AAT	ATG	TTG	ACT	CA	16	408
P90w52	AAT	TTG	TTG	ACT	CAG	15	409	
P90w53	GA	AAT	TTG	TTG	ACT	CA	16	410
P90w54	AAT	ATG	TTG	ACC	CAG	15	411	
P90w55	A	AAT	ATG	TTG	ACC	CA	15	412
P90m56	AAT	ATG	ATG	ACC	CAG	15	413	
P90m57	A	CAG	ATG	ATG	ACC	CA	15	414
P90w58	AAC	ATG	TTG	ACT	CAG	15	415	
P90w59	A	AAC	ATG	TTG	ACT	CAG	15	416
P90w60		TG	TTG	ACT	CAG	CTT	14	417
P90w61		CTG	TTG	ACT	CAG	CTG	14	418
P90m62		CT	ATG	ACT	CAG	CTT	14	419
P90m63		CTG	ATG	ACT	CAG	C-G	14	420
P90w64		TG	ACT	ACA	CAG	CTT	14	421
P90w65		CTG	TTG	ACA	CAG	C-G	14	422
P90w66	AAT	CTG	TTG	ACA	CAG		15	423
P90w67	AAC	CTG	TTG	ACT	CA		13	424
P90w68	A	AAC	CTG	TTG	ACT	C	13	425
P90w69	GA	AAC	CTG	TTG	ACT		13	426
p90w70		TG	TTG	ACT	CAG	ATT G	15	427
p90w71		TG	TTG	ACT	CAG	ATT GGG	16	428
p90w72		G	TTG	ACT	CAG	ATT GGG	15	429
p90w73		TG	TTG	ACA	CAG	CTT G	15	430
p90w74		CTG	TTG	ACA	CAG	CTT	15	431
p90w75		G	TTG	ACA	CAG	CTT GGG	15	432
p90w76		TG	TTG	ACT	CAG	CTT G	15	433
p90w77		G	TTG	ACT	CAG	ATG	15	434
p90w78		G	TTG	ACT	CAG	CTT G	14	435
p90w79		TG	TTG	ACC	CAG	ATT G	15	436
p90w80		G	TTG	ACC	CAG	ATT G	14	437
p90w81		G	TTG	ACC	CAG	ATT GGG	15	438
p90m82		TG	ATG	ACT	CAG	ATT G	15	439
p90m83		TG	ATG	ACT	CAG	ATT GGG	16	440
p90m84		G	ATG	ACT	CAG	ATT GGG	15	441
p90m85		G	ATG	ACT	CAG	ATT GGT	16	442
p90m86		CTG	ATG	ACT	CAG	CTT	15	443
p90m87		TG	ATG	ACT	CAG	CTT G	15	444
P90w88	A	AAT	CTG	TTG	ACT	CA	15	445
P90w89	A	AAT	CTG	TTG	ACT	CA	15	446
p90w90	A	AAT	CTG	TTG	ACT	CA	15	447
p90w100	AAT	CTG	ATG	ACT	CAG		15	448
p90m92	A	AAT	CTG	ATG	ACT	CA	16	449
p90m93	GA	AAT	CTG	ATG	ACT	C	15	450
p90m94		CTG	ATG	ACT	CAG	ATG	15	451
p90m95	AGA	AAT	ATG	ATG			15	452
p90m96	A	AGA	AAT	ATG	ATG	ACT	16	453

**Table 3 - Cont'd-12**

p90m97	A AGA AAT CTG ATG ACT	16	454
p90m98	A AGA AAT ATA ATG ACT	16	455
p90m99	A AAT ATA ATG ACT CAG	16	456
p90m100	AAT ATG ATG ACC CAG	15	457
p90m101	AAC CTG ATG ACT CAG	15	458
p90m102	AGA AAT TTG ATG ACT C	16	459
p90m103	A AAT TTG ATG ACT ATG ACT	16	460
p90m104	AC CTG ATG ACT CAG	14	461
p90m105	AAT CTG ATG ACT CAG A	16	462
p90m106	AT CTG ATG ACT CAG ATG	16	463
p90m107	AT CTG ATG ACT CAG	14	464
p90m108	CTG ATG ACT CAG ATT G	16	465
p90m109	AGA AAT CTG ATG ACT C	16	466
p90m110	AGA AAT CTG ATG ACT	15	467
p90m111	GA AGA AAT CTG ATG A	15	468
p90m112	GGA AGA AAT CTG ATG A	16	469
p90m113	GA AGA AAT CTG ATG AC	16	470
p90m114	AGA AAT CTG ATG AC	14	471
p90w115	AAT CTG TTA ACT CAG	15	472
p90w116	T CTG TTA ACT CAG ATT	16	473
p90w117	AT CTG TTA ACT CAG AG	15	474
p90w118	AGA AAT TTG TTG ACT	16	475
p90w119	GA AAT TTG TTG ACT C	15	476
p90w120	AAT TTG TTG ACT CAG	15	477

Table 5

probes for codon p30	Type B	non-B	probes for codon p48	Type B	non-B	probes for codon p50	Type B	non-B
w25	95.7	98	w47	71.3	70	w31	95.7	98
w29	1.1	0	w45	11.7	22	w44	1.1	2
w32	1.1	1	w72	16	4	w52	8.5	4
w36	1.1	0	m41	3.2	0	m37	1.1	6
m23	1.1	0	neg.	0	8	neg.	1.1	0
neg.	0	1						

Table 5 - Cont'd

probes for codon p54	Type B	non-B	probes for codon p82/84	Type B	non-B	probes for codon p90	Type B	non-B
w3	71.3	48	w91	81.9	70	w27	50	2.5
w34	81.9	62	w60	2.1	12	w37	66.1	17.5
w14	3.2	18	w111	1.1	0	w39	7.1	0
w19	6.4	0	w89	1.1	10	w50	12.5	65
w22	4.3	8	w42	4.3	2	w52	7.1	2.5
w26	0	4	m36	2.1	0	w69	5.4	2.5
w27	0	4	m67	1.1	0	w73	5.4	22.5
m55	3.2	0	m38	2.1	2	w79	0	10
m35	14.9	4	m105	1.1	0	m43	19.6	5
m37	1.1	4	m127	1.1	0	m56	0	2.5
neg.	0	4	m40	14.9	2	neg.	3.6	12.5
			m63	3.2	2			
			m101	2.1	12		3.2	8
			neg.					

**Table 6**

	USA	France	U.K.	Brazil	Spain	Luxemb.	Belgium
p30							
w25	98.9	99.4	88.9	98.3	94.3	100.0	97.0
w29	2.5	0.6	0.0	1.7	0.0	0.0	0.0
w32	3.3	0.6	5.6	5.2	5.7	6.7	1.5
w36	2.5	0.0	0.0	3.4	0.0	0.0	1.0
m23	3.1	0.0	0.0	0.0	0.0	0.0	1.0
neg.	0.6	0.6	5.6	0.0	0.0	0.0	1.0
p46/48							
w47	94.2	80.5	83.3	89.7	97.1	73.3	82.9
w45	8.6	15.6	0.0	1.7	5.7	6.7	11.1
w72	4.2	0.0	16.7	0.0	2.9	13.3	5.0
m41	0.0	0.0	0.0	10.3	0.0	13.3	1.0
neg.	2.8	4.5	0.0	0.0	0.0	0.0	2.5
p50							
w31	96.4	97.4	100.0	96.6	100.0	100.0	96.5
w44	1.7	0.6	0.0	1.7	0.0	0.0	1.0
w52	10.0	4.5	0.0	1.7	2.9	6.7	9.0
m37	2.5	0.6	0.0	1.7	0.0	6.7	0.5
neg.	3.1	2.6	0.0	3.4	0.0	0.0	1.5
p54							
w34	96.9	82.5	97.2	87.9	100.0	53.3	89.4
w3	84.7	77.9	94.4	69.0	82.9	46.7	76.9
w14	3.3	5.8	0.0	3.4	11.4	0.0	6.5
w19	9.2	2.6	0.0	1.7	2.9	6.7	5.5
w22	2.8	10.4	0.0	0.0	5.7	0.0	2.5
w26	0.0	1.3	0.0	0.0	0.0	0.0	0.0
w27	0.0	1.9	0.0	0.0	0.0	0.0	0.5
m55	0.0	0.0	0.0	0.0	0.0	13.3	0.5
m35	1.1	0.0	2.8	6.9	0.0	46.7	3.0
m37	0.0	0.0	0.0	0.0	0.0	13.3	0.0
neg.	0.6	1.3	0.0	1.7	0.0	0.0	2.0
p82/84							
w91	91.6	93.5	94.4	77.6	100.0	73.3	85.9
w60	6.4	2.6	0.0	1.7	2.9	13.3	5.5
w111	3.6	0.6	0.0	1.7	0.0	0.0	0.5
w89	7.0	1.9	0.0	3.4	0.0	0.0	3.0
w42	0.6	0.0	2.8	1.7	0.0	0.0	2.0
m36	0.3	0.0	0.0	0.0	0.0	0.0	0.0
m67	0.0	0.0	0.0	0.0	0.0	0.0	0.5

**Table 6 - Cont'd**

m38	0.0	0.0	0.0	0.0	0.0	6.7	0.0
m105	0.0	0.0	0.0	0.0	0.0	0.0	0.0
m127	0.0	0.0	0.0	0.0	0.0	0.0	0.0
m40	2.8	0.0	8.3	3.4	5.7	46.7	0.0
m63	0.3	0.0	0.0	1.7	2.9	13.3	0.5
m101	1.9	4.5	0.0	3.4	0.0	6.7	4.0
neg.	2.5	3.9	0.0	13.8	0.0	6.7	5.0
p90	USA	France	U.K.	Brazil	Spain	Belgium	
w27	51.1	45.5	34.3	47.7	52.8	25.7	
w37	91.9	73.4	80.0	81.8	88.9	55.2	
w39	0.0	0.0	0.0	0.0	0.0	2.9	
w50	2.6	23.8	2.9	13.6	11.1	21.9	
w52	8.4	11.2	5.7	6.8	13.9	4.8	
w69	5.2	1.4	5.7	2.3	0.0	3.8	
w73	6.1	9.1	0.0	0.0	8.3	6.7	
w79	7.1	11.2	8.6	9.1	5.6	5.7	
m43	1.9	0.0	11.4	0.0	0.0	8.6	
m56	0.3	1.4	0.0	0.0	0.0	0.0	
neg.	1.0	0.0	0.0	0.0	0.0	18.1	

Table 7

Tm lengte Seq ID

pc50w5	AGG	GGG	AAT	TGG	AGG	TTT	TA			20	511			
	26	27	28	29	30	31	32	33	34	35				
pc30w25	ACA	GGA	GCA	GAT	GAT	ACA	GTA	TTA	GAA	GAA				
			GCA	GAT	GAT	ACA	GT				40			
pc30w29			A	GCG	GAT	GAT	ACA				14			
pc30w32				GCA	GAT	GAC	ACA	GT			36			
pc30w36				GCA	GAC	GAT	ACA	GG			14			
pc30m23				A	GCA	GAT	AAT	ACA	GT		42			
	44	45	46	47	48	49	50	51	52					
pc48w37	CCA	AAA	ATG	ATA	GGG	GGA	ATT	GGA	GGT					
				ATG	ATA	GGG	GGA	ATT			15			
pc48w47			AAA	ATG	ATA	GGG	GGA				512			
pc48w73			A	AGA	ATG	ATA	GGG	G			93			
pc48w45				AAA	ATG	ATA	GGA	GGA	ATT		513			
pc48w72				A	AAA	ATA	ATA	GGG	GGA		42			
pc48m41						ATG	ATA	GTG	GGA	ATT	18			
											91			
	48	49	50	51	52	53	54				120			
pc50w31	GGG	GGA	ATT	GGA	GGT	TTT	ATC				87			
pc50w44			GGG	ATT	GGA	GGT	TTT				151			
pc50w52				GGG	ATT	GGG	GGT	TT			164			
pc50m37				GA	ATT	GGA	GGC	TTG			14			
	GGG	GGA	GTT	GGA							172			
											157			
	51	52	53	54	55	56	57	58						
pc54w34	GGA	GGT	TTT	ATC	AAA	GTA	AGA	CAG						
		GA	GGT	TTT	ATC	AAA	GT				212			
pc54w14			GGT	TTT	ATC	AAG	GTA	A			189			
pc54w19			A	GGC	TTT	ATC	AAA	GTA			194			
pc54w22				GA	GGT	TTT	ATT	AAA	GTA		197			
pc54w26				A	GGT	TTC	ATT	AAG	GTA		202			
pc54w27					GGT	TTT	ATT	AAG	GTA	A	204			
pc54m35						GGT	TTT	GTC	AAA	GTA	15			
pc54m37							GGT	TTT	GTC	AGA	213			
pc54m55								GGT	TTT	GTC	215			
	A	GGT	TTT	GCC	AAA	GT					516			
	78	79	80	81	82	83	84	85	86	87				
pc82w91	GGA	CCT	ACA	CCT	GTC	AAC	ATA	ATT	GGA	AGA				
			ACA	CCT	GTC	AAC	ATA	A						
pc82w60				CA	CCT	GTC	AAT	ATA	ATG		44			
pc82w111					A	CCG	GTC	AAC	ATA	ATT	16			
pc82w89						ACA	CCT	GTT	AAC	ATA	338			
pc82m101							ACA	CCT	ATC	AAC	AG	17		
pc82w42								ACA	CCT	ATC	AT	316		
pc82m38									CA	CCT	GTC	269		
pc82m105										ACA	CCT	265		
pc82m127											ACG	CCC	332	
											CA	CCT	354	
											TTC	AAC	GT	ATG

**Table 7 - Cont'd**

pc82m40	ACA	CCT	GCC	AAC	ATA		44	15	267		
pc82m63	CA	CCT	GCC	AAT	ATA	AG	42	16	290		
pc82m36	ACA	CCT	ACC	AAC	ATA			15	518		
pc82m67	ACA	CCT	ACC	AAC	GT		14	519			
	86	87	88	89	90	91	92	93	94		
	GGA	AGA	AAT	CTG	TTG	ACT	CAG	ATT	GGT		
pc90w27	AAT	CTG	TTG	ACT	CA			38	14	384	
pc90w37	T	CTG	TTG	ACT	CAG	AT			15	514	
pc90w39	GA	GTC	AAC	AGA	GTT	C			15	515	
pc90w50	AAT	ATG	TTG	ACT	CAG			40	15	407	
pc90w52	AAT	TTG	TTG	ACT	CAG			40	15	409	
pc90w69	GA	AAC	CTG	TTG	ACT			40	14	426	
pc90w73		TG	TTG	ACA	CAG	CTT	G		44	15	430
pc90w79		TG	TTG	ACC	CAG	ATT	G		44	15	436
pc90m138	GTC	ATC	AGA	TTT	CT				14	510	
pc90m56	AAT	ATG	ATG	ACC	CAG			42	15	413	